Serotoninergic Control of Adrenal Zona Glomerulosa Function.

by

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Declaration

This thesis was composed by myself, and the work presented in it is entirely my own. Measurements made by others in collaborative studies and figures used from other sources are acknowledged and indicated in the text.

Eleanor Davies

Dedication

This thesis is dedicated to my Mum, Dad and Sister .

Abbreviations

AII	-	Angiotensin II
AIII	-	Angiotensin III
ACE	-	Angiotensin converting enzyme
ACTH	-	Adrenocorticotrophic hormone
ANF	-	Atrial natriuretic factor
APUD	-	Amine precursor uptake and decarboxylation
ASF	-	Aldosterone stimulating factor
ATP	-	Adenosine tri-phosphate
AVP	-	Arginine vasopressin
BAL	-	British anti-leucite
BSA	-	Bovine serum albumin
BOL	-	2-Bromolysergic acid
Ca^{2+}	-	Calcium ion
$Ca\ Cl_2$	-	Calcium chloride
CAP	-	Captopril
Ci	-	Curie
CNS	-	Central nervous system
Cpm	-	Counts per minute
CRF	-	Corticotrophin releasing factor
Cyclic AMP	-	Adenosine 3',5'-cyclic monophosphate
Cyclic GMP	-	Guanosine 3',5'-cyclic monophosphate
DA	-	Dopamine
DAG	-	Sn-1,2,-diacyl glycerol
DARS	-	Donkey-anti rabbit serum
db cyclic AMP	-	Dibutyryladenosine 3',5'-cyclic monophosphate
DEX	-	Dexamethasone
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
EDTA	-	1,2-Di (2-aminoethoxy) ethane tetraacetic acid
EGTA	-	Ethyleneglycol-bis (β-aminoethyl ether)-N,N,N',N-tetraacetic acid
ER	-	Endoplasmic reticulum
EtOH	-	Ethanol
GDP	-	Guanosine di-phosphate
G_i	-	Inhibitory G protein
G_s	-	Stimulatory G protein
GTP	-	Guanosine tri-phosphate
^3H	-	Tritiated
HCl	-	Hydrochloric acid

HDL	-	High density lipoprotein
5HIAA	-	5-Hydroxyindoleacetic acid
Hr	-	Hour
5HT	-	5-Hydroxytryptamine ; serotonin
5HTP	-	5-Hydroxytryptophan
HPLC	-	High pressure liquid chromatography
^{125}-I	-	Iodinated
IP	-	Inositol mono-phosphate
IP_3	-	Inositol 1,4,5-triphosphate
i.p	-	Intra-peritoneal
JG	-	Juxtaglomerular
K+	-	Potassium ion
KCl	-	Potassium chloride
K_D	-	Dissociation constant
KH_2PO_4	-	Potassium dihydrogen phosphate
K_m	-	Michaelis constant
LDL	-	Low density lipoprotein
LSD	-	Lysergic acid diethylamide
MABP	-	Mean arterial blood pressure
µ	-	Micron
µg	-	Microgram
µl	-	Microlitre
µM	-	Micromolar
M	-	Molar
ml	-	Millilitre
mg	-	Milligram
$MgSO_4$	-	Magnesium sulphate
Min	-	Minute
mM	-	Millimolar
MSH	-	Melanocyte stimulating hormone
Na+	-	Sodium ion
Na Cl	-	Sodium chloride
NAD+	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate
ng	-	Nanogram
Na_2HPO_4	-	Di-Sodium hydrogen phosphate
NaH_2PO_4	-	Sodium di-hydrogen phosphate
NaOH	-	Sodium hydroxide
NaN_3	-	Sodium azide
NIRS	-	Non-immune rabbit serum
nM	-	Nanomolar

PCPA	-	Para-chlorophenylalanine
PI	-	Phosphatidylinositol
PIP_2	-	Phosphatidylinositol 4,5-bisphosphate
pM	-	Picomolar
POMC	-	Pro-opiomelanocortin
PRA	-	Plasma renin activity
RAS	-	Renin-angiotensin system
RIA	-	Radioimmunoassay
RNA	-	Ribonucleic acid
SEM	-	Standard error of mean
SHR	-	Spontaneously hypertensive rat
TFP	-	Trifluoperazine
TMB-8	-	8-(Diethylamino) octyl 3,4,5-trimethoxybenzoate
TPA	-	12-O-Tetradecanoyl phorbol-13-acetate
VLDL	-	Very low density lipoproteins
V/V	-	Volume for volume
W/V	-	Weight for volume
W/W	-	Weight for weight
ZF	-	Zona fasciculata
ZG	-	Zona glomerulosa
ZR	-	Zona reticularis

Abstract

A clearer understanding of the physiological mechanisms controlling aldosterone secretion is likely to be of major importance in appreciating the significance of the abnormalities found in patients with conditions such as essential hypertension.

Although angiotensin II, adrenocorticotrophic hormone (ACTH) and potassium are considered to be the major physiological regulators of aldosterone secretion, serotonin (5-hydroxytryptamine, 5HT) is a potent stimulus for aldosterone secretion both *in vivo* and *in vitro* in man and the rat. However, the physiological and pathophysiological role of serotonin in the control of mineralocorticoid secretion remains unclear. Unlike the cardiovascular and central nervous system (CNS), where specific serotonin receptors have been identified and categorised into the $5HT_{1a}$, $5HT_{1b}$, $5HT_{1c}$, $5HT_{1d}$, $5HT_2$ and $5HT_3$ sub-types, specific receptors for serotonin in the adrenal zona glomerulosa and the second messenger system to which they are coupled, have yet to be formally characterised. To resolve this, the effects of the selective serotonin receptor antagonists ketanserin ($5HT_2$), methysergide ($5HT_{1/2}$), mesulergine ($5HT_{1c/2}$), cyanopindolol ($5HT_{1a/1b}$) and ICS 205/930 ($5HT_3$) have been studied on the aldosterone response to serotonin in isolated rat adrenal zona glomerulosa cells. The specificity of the antagonists was also investigated by observing the effects of the drugs on the aldosterone response to angiotensin II, ACTH and potassium. The signal transduction mechanism for serotonin in the zona glomerulosa was studied by measuring cyclic AMP and phosphatidylinositol (PI) turnover and comparing the results with those of angiotensin II and ACTH, which act through phospholipase C and adenylate cyclase respectively. In addition, the role of calcium was investigated using the chelating agent EGTA, the calcium channel inhibitors verapamil and nifedipine, the intracellular calcium channel blocker TMB-8, and the calmodulin antagonist trifluoperazine (TFP). Transmembrane calcium flux in response to serotonin was also studied directly by radiolabelled calcium influx experiments.

Serotonin produced a dose-dependent increase in cyclic AMP and aldosterone secretion, whilst PI turnover was unaffected. The cyclic AMP and aldosterone responses to serotonin were inhibited by mesulergine, methysergide and ketanserin. The aldosterone response to angiotensin II, but not ACTH or potassium, was also inhibited by these antagonists. Cyanopindolol and ICS 205/930 produced a small inhibition of serotonin stimulated aldosterone and cyclic AMP secretion, but had no effect on the aldosterone response to the other agonists. The presence of EGTA, verapamil, nifedipine, TMB-8 and TFP also significantly inhibited aldosterone secretion in response to serotonin. Radiolabelled calcium influx was stimulated by serotonin and this could be blocked by verapamil.

In addition to the *in vitro* studies, two *in vivo* studies were also carried out. The effect of acute serotonin enhancement was studied in rats with indwelling arterial cannulae. This experimental model avoided the use of anaesthetics which can activate the renin-angiotensin system (RAS). Plasma aldosterone was measured prior to and after administration of the immediate precursor to serotonin, 5-hydroxytryptophan (5HTP). Aldosterone was elevated 45 minutes after administration of 5HTP, and this could be blocked, though not completely, by pretreatment with both dexamethasone and captopril. The inhibitory effect of captopril could be reversed by co-administration of angiotensin II. The effect of chronic serotonin enhancement on adrenal zona glomerulosa growth was studied in rats given 5HTP for periods of up to 2 weeks. 5HTP increased the width of the zona glomerulosa, although the effect was less than that observed with sodium depletion. No changes were detected in plasma renin activity (PRA), angiotensin II, corticosterone or aldosterone and the trophic effect could not be reversed by chronic treatment with captopril or dexamethasone.

In conclusion, the aldosterone response to serotonin in the zona glomerulosa appears to be mediated predominantly by $5HT_{1C}$ / $5HT_2$ like receptors which modulate the steroid response to angiotensin II and, in contrast to other serotonin responsive tissues, couple to the adenylate cyclase second messenger system. In addition, influx of extracellular calcium, which may act cooperatively with cyclic AMP to activate the cascade mechanism resulting in

steroidogenesis, is necessary for the action of serotonin. *In vivo* it appears that a number of different mechanisms mediate the acute and chronic actions of serotonin. Acutely, serotonin activates the hypothalamo-pituitary adrenal axis and the renin-angiotensin system, both of which require to be intact for the full aldosterone response, although angiotensin II appears to act purely in a permissive capacity. It is also likely that there is a direct action of serotonin on the adrenal cortex. In contrast, chronic serotonin enhancement has no effect on the renin-angiotensin system, the hypothalamo-pituitary adrenal axis or steroidogenesis, although zona glomerulosa growth is stimulated, most probably by a direct action of serotonin, although the possibility that serotonin could stimulate the production of another growth factor(s), cannot be excluded at this stage. Therefore, chronically, there appears to be an "escape" from continued mineralocorticoid augmentation by an as yet unidentified mechanism.

The specific interaction between serotonin, the angiotensin II receptor, the renin-angiotensin system and the hypothalamo-pituitary adrenal axis may be important regulatory components of mineralocorticoid secretion, and any disturbance in this fine balance may lead to alterations in steroid secretion. Further studies are required to investigate more fully the importance of serotonin in the physiology and pathophysiology of the adrenal cortex.

Chapter One

The Adrenal Gland and Aldosterone Secretion.

1.1. Introduction

This chapter serves as a brief review of the adrenal gland, describing its structure, products and their function. However, in parallel with the theme of this thesis particular emphasis is placed on the biosynthesis, metabolism, function and the secretory control of the principal product of the zona glomerulosa, the mineralocorticoid aldosterone.

1.2. Topography and embryology of the adrenal gland

All higher vertebrates including man possess two pyramidal shaped adrenal glands which are located in the fatty tissue on the upper pole of each kidney. A section across the gland reveals two clearly distinct regions, an inner yellowish coloured core, the medulla, surrounded by a dark red coloured cortex. The cells of the cortex originate from mesodermal tissue and are associated with steroid secretion, whilst those of the medulla stem from ectodermal cells which differentiate to form either mature ganglionic cells or the chromaffin cells which secrete catecholamines. (Crowder 1957)

Each gland is supplied by a large volume of blood arising from several groups of arteries which branch into an arteriole network as they approach the gland. After crossing the gland they converge in the medulla which, in man, drains into the inferior vena cava from the right adrenal and into the renal vein from the left adrenal. This expansive network of blood vessels ensures a continuous supply of oxygen, co-factors and precursors, each necessary to maintain the steroidogenic capacity of the gland, as well as providing a rapid clearance system of the steroids from the adrenal, which is one of the few glands which does not store its own products. (Gagnon 1957)

1.3. Histology

The adrenal cortex comprises three readily recognisable cell types which are arranged in concentric zones or layers (Figure 1.1). Each of the zones has a specific function and is involved in the biosynthesis of one or more corticosteroids and different control mechanisms

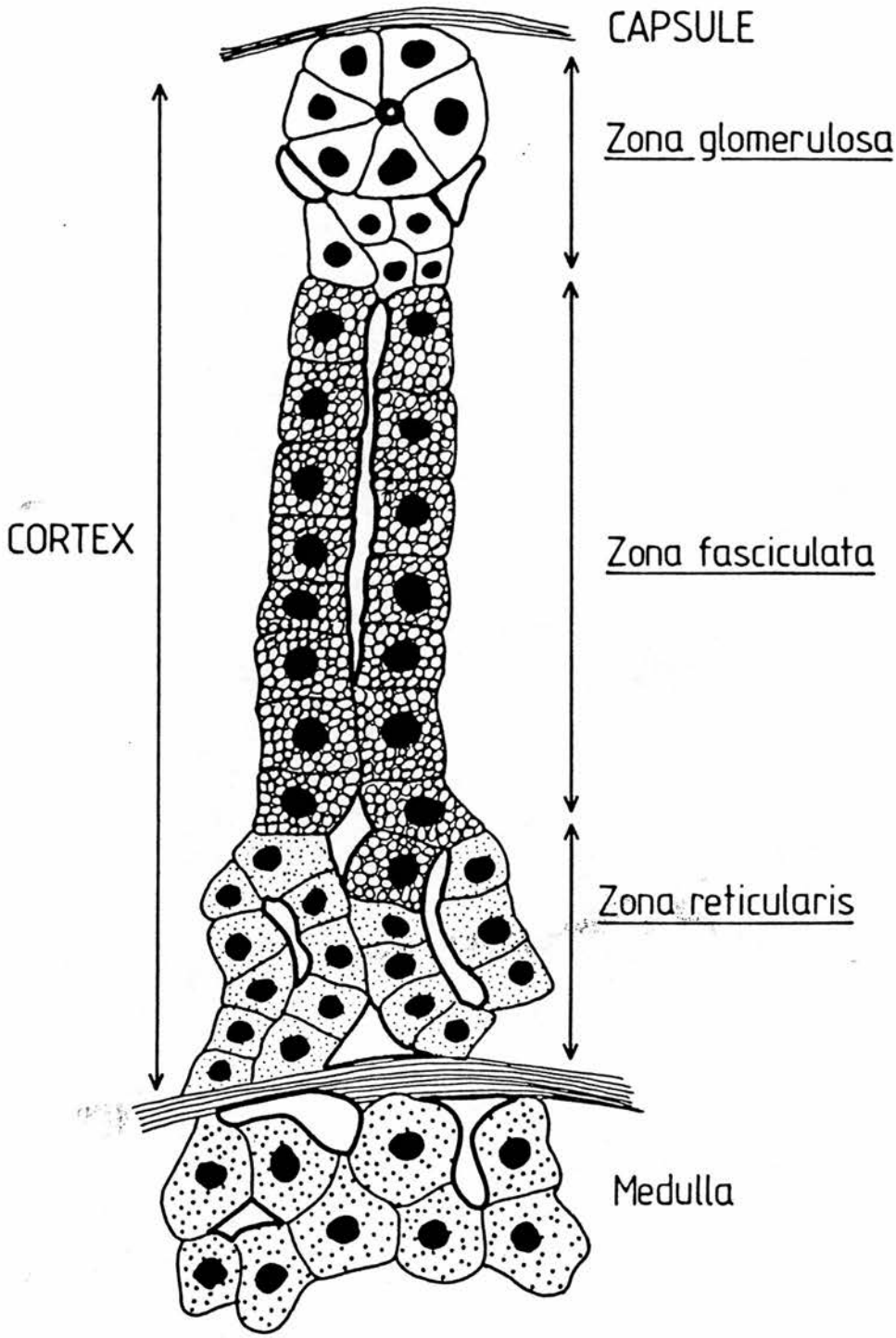


Figure 1.1. The histology of the adrenal gland

are exerted depending on the zone, stimulus and end product. The outermost zona glomerulosa, whose component cells have a high nuclear : cytoplasmic ratio, aggregate to form compact ball-like clusters. They also have an abundance of smooth endoplasmic reticulum (E.R), and very little rough E.R. The intermediate zona fasciculata is the largest of the zones and the cells are arranged in radially disposed columns extending from the glomerulosa. They contain a large amount of smooth E.R and ascorbic acid and an abundance of lipid. Lastly, the innermost zona reticularis cells are low in lipid content and are arranged in a network system. The cortex surrounds the adrenal medulla which is composed of columnar shaped chromaffin cells, individually innervated by cholinergic preganglionic neurones and full of membrane-bounded electron dense granules which are thought to be the main storage sites of the catecholamines adrenaline and noradrenaline. The three zones of the cortex and the inner medulla are encased by a capsule composed of several layers of elongated connective tissue cells surrounded by collagen fibres. (Dobbie *et al* 1966) (Elias and Pauly 1956)

1.4. Steroid Biosynthesis

The adrenal cortex synthesises at least 50 different steroids, each possessing a basic 4 ringed structure and a varying number of carbon atoms from which a number of substitutions, e.g ketones, aldehydes and hydroxyls, may extend either above (β) or below (α) the plane of the central ring structure. Many of the steroids have no hormonal activity and serve only as intermediates or precursors to the main steroids, which can be divided into the 5 main groups outlined below and illustrated in Figure 1.2.

1. Progestogens e.g progesterone contain 21 carbon atoms and are involved in the preparation of the lining of the uterus for implantation of an ovum.

2. Androgens e.g testosterone contain 19 carbon atoms and have a ketone group on the carbon position 17. These are responsible for the development of male secondary sex characteristics.

3. Oestrogens e.g oestradiol contain 18 carbon atoms and cause the development of female

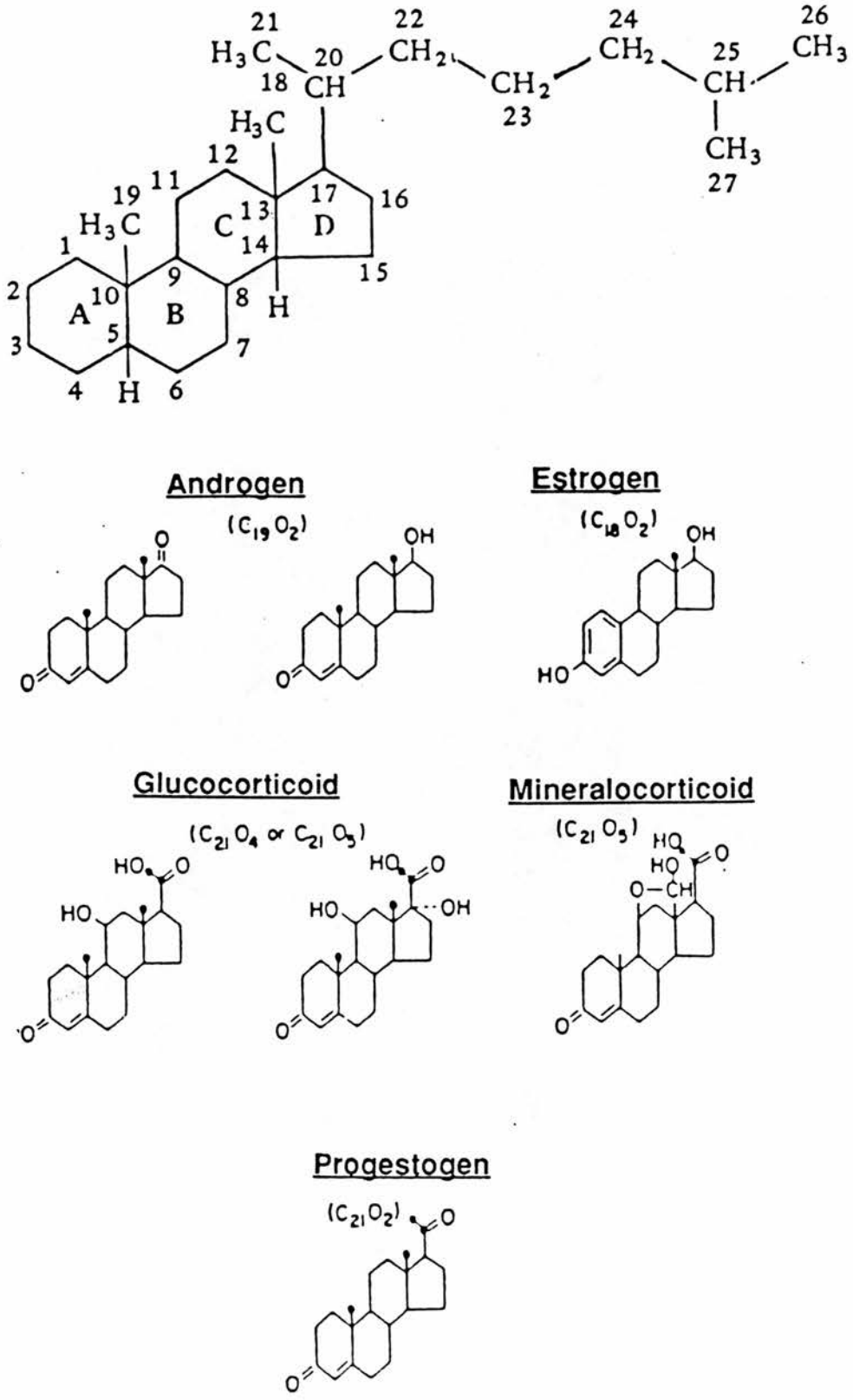


Figure 1.2. The 5 main steroid groups and their numbering system.

secondary sex characteristics.

4. Glucocorticoids e.g cortisol in man and corticosterone in the rat contain 21 carbon atoms and an OH group at the 17 carbon position. These enhance gluconeogenesis and glycogen formation in addition to degradation of fat and protein.

5. Mineralocorticoids e.g aldosterone contain 21 carbon atoms and cause reabsorption of Na^+, Cl^- and HCO_3^- by the distal tubule of the kidney.

All of the steroids synthesised in the adrenal are derived from cholesterol, which is either synthesised by the adrenal cortex and other tissues or derived from animal fat in the diet. Cholesterol is stored in close association with plasma lipoproteins e.g low density lipoproteins (LDL), very low density lipoproteins (VLDL) and high density lipoproteins (HDL), and of these, the cholesterol in co-existence with LDL appears to be the preferential substrate for steroid biosynthesis. (Borkowski *et al* 1972) (Carr *et al* 1980)

LDL binds to specific receptors located on the adrenal cortical cell surface forming a complex which is internalised and hydrolysed in lysosomes to release free cholesterol, part of which is esterified and utilised as a substrate at a later stage whilst the remainder is used directly for steroid biosynthesis.

The initial step in the biosynthetic pathway (Figure1.3) is the conversion of the liberated cholesterol to Δ-5-pregnenolone. (Whitehouse and Vinson 1968) This side chain cleavage step consists of several reactions, catalysed by the cholesterol desmolase enzyme complex which is located in the mitochondria and requires molecular oxygen and NADPH as co-factors. The complex comprises an electron transport chain with the flavoprotein adrenodoxin reductase, an iron sulphur protein adrenodoxin and a reaction specific cytochrome P_{450}, which is just one of a large family of cytochromes catalysing hydroxylations. There are at least 4 different types of cytochrome P_{450}, these include P_{450}-17α, P_{450} -side chain cleavage (scc), P_{450} -C21 and P_{450}-11β. (Simpson and Waterman 1988) Hydroxylation occurs when electrons reduce the cytochrome P_{450} after passing along the electron transport chain. This reaction reduces molecular oxygen to a molecule of water and an atom of oxygen which is placed on the appropriate hydrogen moiety.

The resulting pregnenolone passes to the endoplasmic

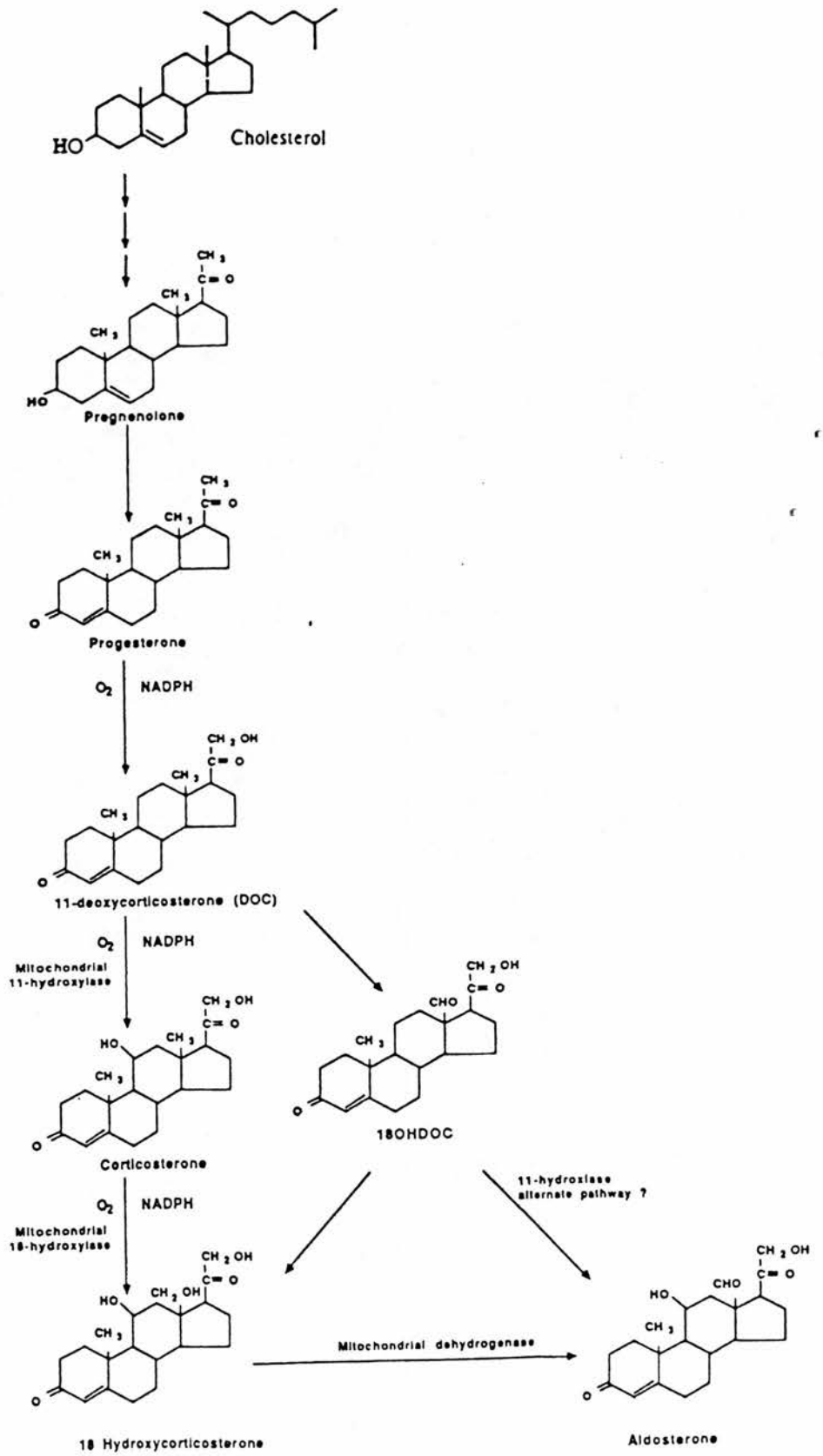


Figure 1.3. **The aldosterone biosynthetic pathway.**

reticulum where it is channelled into synthesis of the appropriate corticosteroid. This initial side chain cleavage reaction is considered to be the rate limiting step in all the steroidogenic pathways and is consequently under the control of a number of different factors e.g angiotensin II, ACTH and serotonin.

Cortisol, the major glucocorticoid in man is formed by either 3-hydroxysteroid dehydrogenation and isomerisation of pregnenolone to form progesterone, followed by the sequential hydroxylation of the 17, 21 and 11 carbon atoms to form 17α-OH progesterone, 11-deoxycortisol and cortisol respectively or 17α-hydroxylation of pregnenolone to form 17α-OH-pregnenolone preceding the dehydrogenation/isomerisation reaction forming 17α-OH-progesterone and then 21 and 11 hydroxylation as before. Alternatively progesterone can also be hydroxylated at the 21 and 11 carbon position to form (Cameron *et al* 1968)
11-deoxycorticosterone and corticosterone respectively. The latter compound is the major glucocorticoid in the rat, due to the absence of 17α-hydroxylase activity, which in man is predominant in the zona glomerulosa (Figure 1.4). (Simpson and Mason 1976)

17 and 21 hydroxylations are catalysed by a microsomal enzyme complex consisting of a NADPH-cytochrome P_{450} reductase and the appropriate cytochrome P_{450}. The 11-ß-hydroxylations which take place in the mitochondria are catalysed by another reaction specific cytochrome enzyme similar to that involved in pregnenolone formation. The dehydrogenation/isomerisation reactions are catalysed by 3ß hydroxysteroid dehydrogenase which requires NAD+, and Δ^5-3 ketosteroid isomerase. (Simpson and Mason 1976) (Grant 1978)

Aldosterone, the principal mineralocorticoid in man is formed predominantly from corticosterone via 18-OH-corticosterone by 2 reactions, methyl oxidation 1 and methyl oxidation 2 (Ulick 1976). This reaction involves a cytochrome P_{450} with cytomethyloxidase activity and takes place exclusively in the zona glomerulosa. Alternatively, aldosterone may be formed from 18-OH-deoxycorticosterone, which is synthesised from 11-deoxycorticosterone. The 18-OH-deoxycorticosterone may either be converted to 11-deoxyaldosterone or to 18-OH-corticosterone before conversion to aldosterone (Muller 1980). The conversion of 18-OH-deoxycorticosterone to aldosterone has been shown in

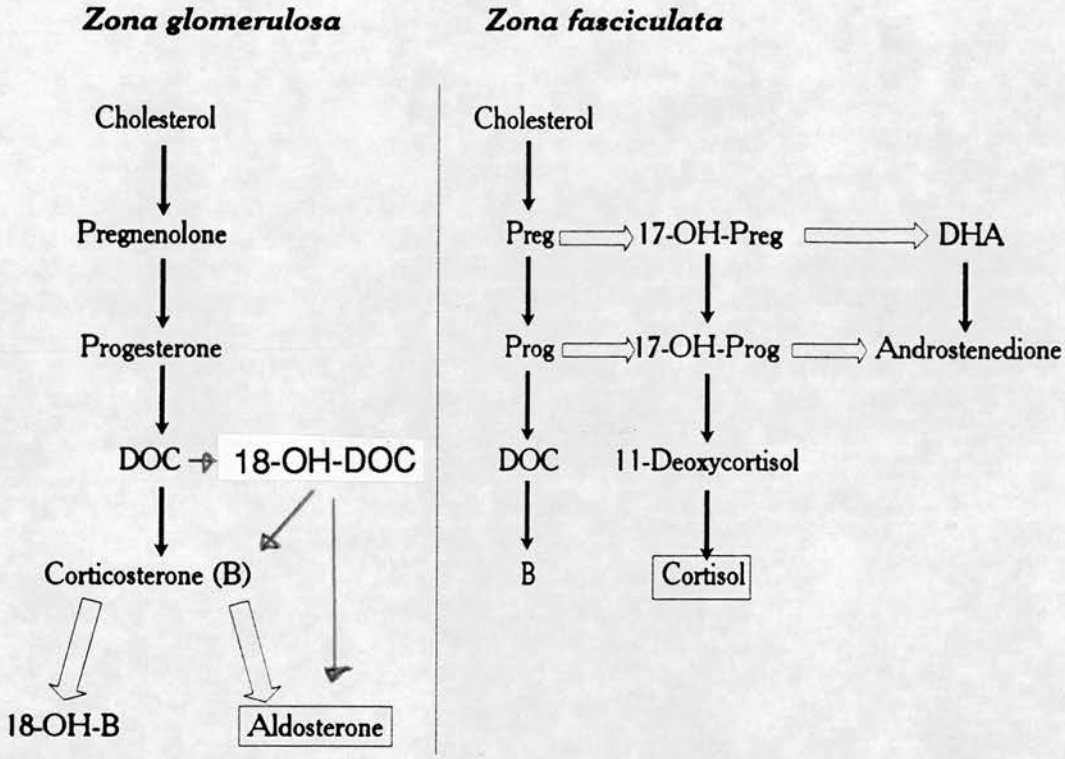


Figure 1.4. **The zonation of adrenal steroidogenesis.**

N.B. The biosynthesis of cortisol does not occur in the rat, corticosterone is the principal glucocorticoid.

(Pasqualini 1964)
normal tissue but in particular in tumourous tissue (Grekin *et al* 1973). The normal and alternative pathways for aldosterone biosynthesis are shown in figure 1.3.

Finally androgens including androstenedione and testosterone and oestrogens e.g oestradiol are synthesised by a similar series of dehydrogenation/isomerisation and hydroxylation reactions.

Two sites in the biosynthetic pathway of aldosterone appear to be rate limiting, the first being the conversion of cholesterol to pregnenolone and the other occurring at a later stage in the pathway, probably between the conversion of corticosterone to aldosterone. A number of controlling factors, which will be discussed in detail in subsequent sections, act on these early and late stages of the pathway to modulate and control aldosterone production.

1.5. Metabolism of steroids

The reactions which render steroids biologically inactive and readily water soluble for excretion in urine or bile can be divided into 5 groups; reductions, hydroxylations, side-chain cleavage, oxidations and esterifications. These reactions are carried out mainly in the liver, but they can also take place in the kidney and the adrenal gland itself. (Bondy 1980)

1.5.1. Reductions

Steroids can be inactivated by removal of the Δ4,3-ketone group. This irreversible reaction is carried out by reducing the double bond between the 4th and 5th carbon atoms to yield a hydrogen atom on the 5th carbon in either the α or ß position. The resulting reduced 19-carbon compounds are termed 5α or 5 β androstanes, whilst the 21 carbon compounds are known as 5α or 5ß pregnanes. Normally, the 19 carbon steroids are preferentially reduced to the α form and the 21 carbon steroids to the ß form. The initial reduction is usually followed by a second reversible reaction which converts the 3-ketone group to a 3-hydroxyl group.

1.5.2. Hydroxylations

Cortisol in particular can be readily excreted by the addition of a hydroxyl group at the 6ß position. This reaction, which takes place in the liver, is particularly important in children and also patients with liver disease. A similar reaction at the 16α position converts oestradiol to estriol.

1.5.3. Side Chain Cleavage

The removal of the carbon 20/21 side chain from 17-hydroxylated steroids such as cortisol occurs after reduction of the A ring and forms a 19 carbon compound with a ketone group on the 17 carbon position.

1.5.4. Oxidations

Two types of oxidation reactions occur. Firstly, the 11ß hydroxyl group of the steroid can be converted to a ketone e.g cortisol to cortisone, a reaction which is reversible providing the A ring remains intact. Secondly, subsequent to ß reduction of the A ring, the hydroxyl group is oxidised to a carboxyl group to produce either the cortolic acids, which are formed when the 11ß-hydroxyl group is intact, or the cortolonic acids if a ketone group is present at the carbon 11 position.

1.5.5. Esterification

21 carbon steroids are esterified with glucoronic acid at the 3ß-hydroxyl position by glucuronyl transferase to form glucuronidates, whilst 19 carbon steroids are converted to sulphates at the 3-hydroxyl position or doubly at the 3 and 21 carbon position.

1.5.6. Metabolism of aldosterone

Aldosterone is metabolised in a number of ways and the key metabolites are shown in Figure 1.5. However, the main route of metabolism takes place in the liver, where reduction of

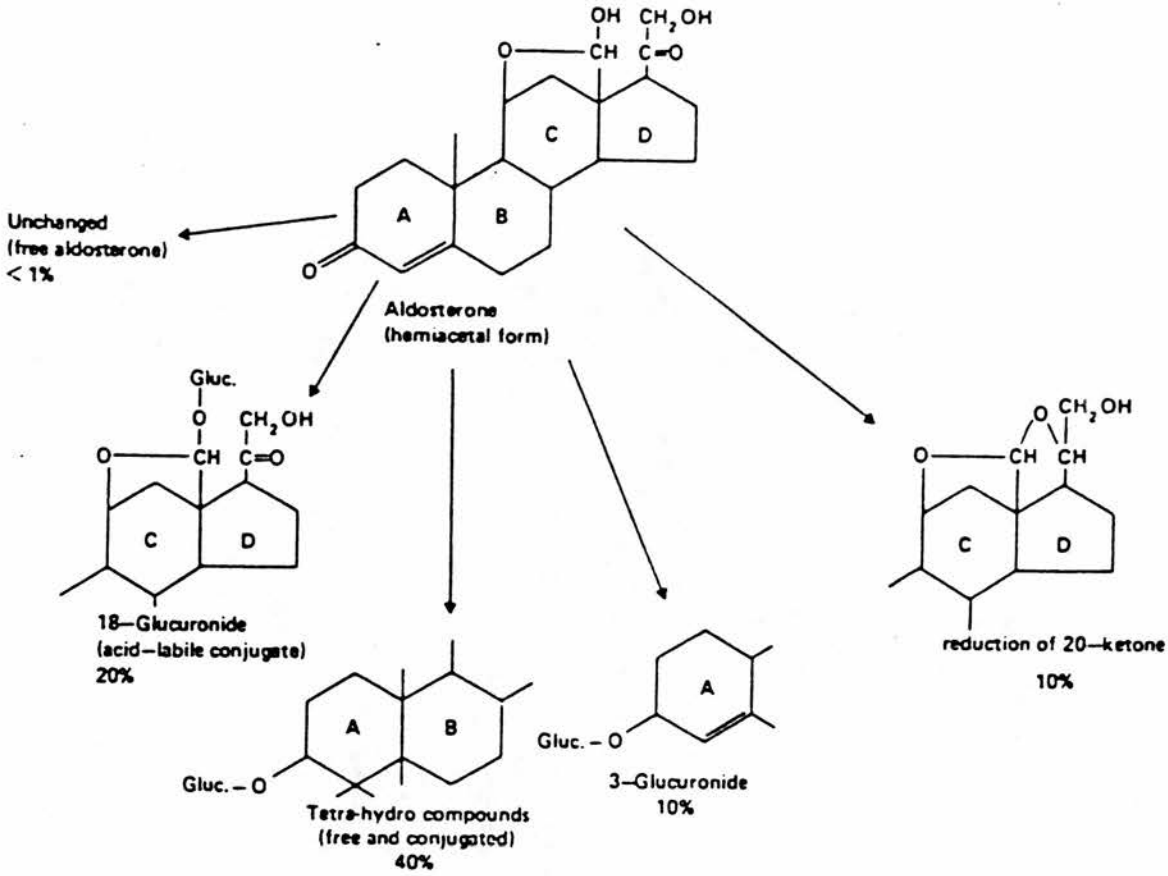


Figure 1.5. **The metabolism of aldosterone.**

the A ring and the 3-ketone group forms tetrahydroaldosterone. This is then esterified at the 3-hydroxyl position with glucuronic acid and subsequently excreted. Alternatively, conjugation of the 18 carbon position with glucoronic acid, without alterations of the A ring and the 3-ketone group, can take place in the kidney. The latter derivative is labile in dilute acid and therefore free aldosterone is released by hydrolysis at pH 1. It is this compound which is referred to when urinary aldosterone measurements are made.

1.6. Actions of aldosterone

As early as the 1940s it was known that altered sodium status affected the width of the zona glomerulosa, implying that this zone secreted a hormone involved in maintaining sodium status. This was thought to be deoxycorticosterone until the 1950s when Simpson and Tait isolated and characterised aldosterone which is now known to be the principal mineralocorticoid in man.

Aldosterone has a short half life of approximately 20-30 minutes, and its circulating concentration is low, i.e 200-800 pM. This may be explained in part by the fact that it is not well protected by carrier proteins or binding globulins, and it is therefore rapidly metabolised by the liver.

Aldosterone acts predominantly on the distal convoluting tubule and the collecting ducts of the kidney, although extra-renal sites of action include the sweat and salivary glands. In the kidney it diffuses into the epithelial cells and binds to highly specific cytoplasmic receptor proteins. This complex moves into the nucleus where it induces transcription of a specific RNA related to sodium / potassium transport. This RNA facilitates protein synthesis in conjunction with the ribosomes located in the cytoplasm. The protein(s) formed are thought to mediate reabsorption of sodium and excretion of potassium by (Figure 1.6);

a. Increasing the activity or production of various mitochondrial enzymes that increase cellular adenosine tri-phosphate (ATP) and therefore enhance the activity of the membrane (Na^+, K^+)-ATP-ase.

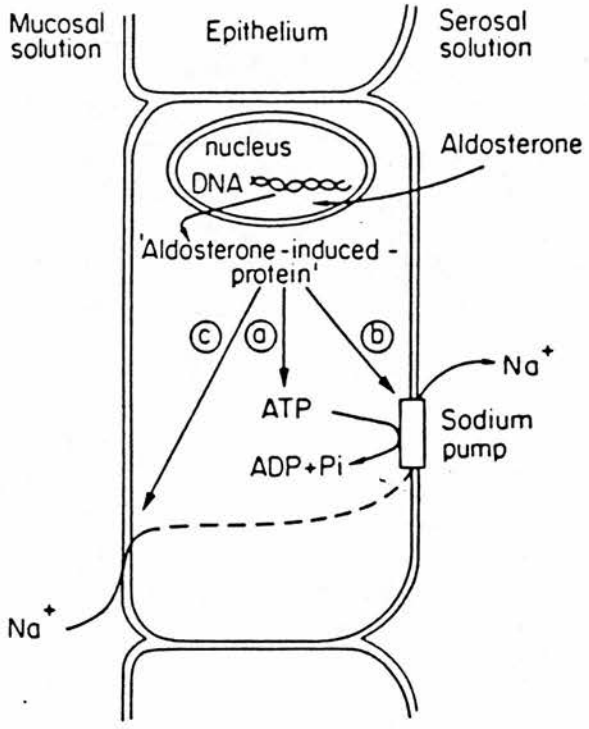


Figure 1.6. **The cellular action of aldosterone.**

b. Increasing the activity of the (Na +, K+)-ATP-ase directly.

c. Increasing the sodium permeability of the cortical collecting tubule apical membrane exposed to the tubular lumen. (Sharp and Leaf 1966)

d. By other as yet unidentified factors. (Edelman and Fimognari 1968) (Marver and Kokko 1983)

The resulting increase in intracellular sodium generates a negative potential which is returned to its resting state by potassium excretion. Hydrogen ion secretion in the medullary collecting tubules is also stimulated. The renal effects of aldosterone commence 1-2 hours after administration, reflecting the time required for protein synthesis, and are sustained for 4-8 hours. As expected mineralocorticoid deficiency causes sodium loss, hyperkalemia and acidosis, whilst excess production results in sodium retention, hypokalemia and alkalosis. (Kassirer *et al* 1970) (Seldin *et al* 1972)

The overall action of aldosterone depends markedly on sodium status. Increased sodium intake results in more tubular sodium available for reabsorption, thus enhancing potassium excretion. Conversely, sodium restriction diminishes both sodium reabsorption and kaliuresis. Potassium itself can stimulate aldosterone secretion and this will be fully discussed in a subsequent section. This effect leads to eventual potassium excretion and serves as an intrinsic part of the bodies defence mechanism against hyperkalemia. An additional mechanism protects against persistent mineralocorticoid excess, whereby the body 'escapes' from further sodium retention and hypokalemia. This is thought to be mediated via secondary increases in other factors, such as atrial natriuretic peptide and renal haemodynamics.

1.7. Control of aldosterone secretion

In order to fully understand the pathophysiology of aldosterone secretion in diseases such as essential hypertension it is necessary to gain a detailed understanding of the mechanisms which control its secretion under normal conditions.

Unlike many other steroids, whose biosynthesis is controlled by a singular mechanism, the regulation of aldosterone production is a complicated multifactorial process involving a

series of contrasting signal-transduction mechanisms, the main ones of which are discussed below. However, although many of these stimulate or inhibit aldosterone secretion both *in vivo* and / or *in vitro*, their physiological importance remain unclear.

1.7.1. The renin-angiotensin system

The renin-angiotensin system is thought to be the major physiological regulatory component of aldosterone secretion, and parallel changes in plasma renin activity (PRA) and aldosterone secretion under a variety of conditions confirm this (Davis *et al* 1975, Fraser *et al* 1979, Coghlan *et al* 1979).

The proteolytic enzyme renin is synthesised by the juxtaglomerular cells of the kidney and stored in intracellular granules. It is released exocytotically in response to a number of stimuli, including a decrease in blood volume, pressure or plasma sodium concentration. Upon its release it diffuses into the circulation where it acts on angiotensinogen, perhaps more commonly known as renin substrate, cleaving it at the leucine-leucine peptide bond to form the biologically inactive angiotensin I. The 2 amino acids from the C-terminal of angiotensin I are then cleaved by angiotensin converting enzyme (ACE), an endopeptidase located in many tissues but predominantly the lung, to form the active octapeptide angiotensin II. This step can be blocked specifically by a group of drugs known as ACE inhibitors and these include captopril and enalapril.

Angiotensin II stimulates aldosterone production in the adrenal glomerulosa both *in vivo* and *in vitro* at both the early and late stages of the biosynthetic pathway (Ganong *et al* 1962, Kaplan 1965, Haning *et al* 1970, Aguilera and Marusic 1971). In addition, it stimulates catecholamine release from the medulla and is also a potent vasopressor, increasing peripheral arteriole resistance and subsequently blood pressure. During sodium depletion the aldosterone response to angiotensin II is enhanced and the glomerular filtration rate is decreased therefore reducing sodium excretion directly. Conversely, during sodium repletion the response to angiotensin II is diminished, resulting in sodium excretion.

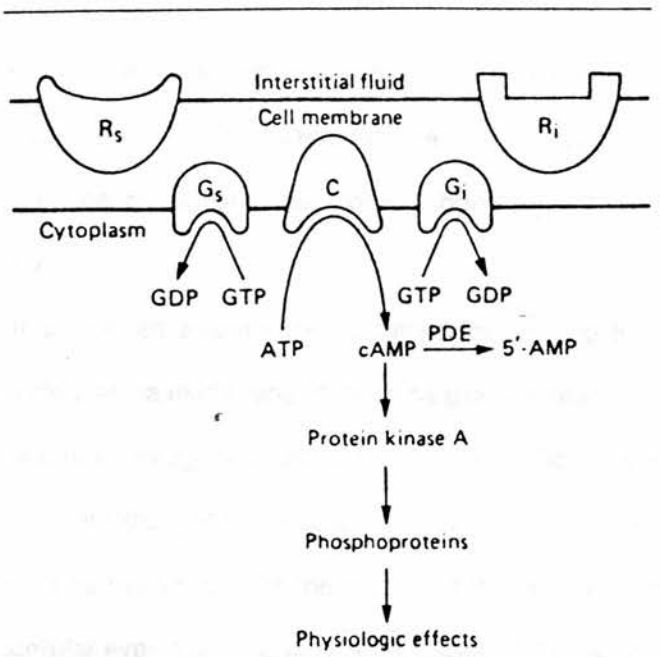


Figure 1.6b. The cyclic AMP system. Activation of the catalytic unit (C), of adenylate cyclase, catalyses the conversion of ATP to cyclic AMP. Cyclic AMP activates protein kinase A, which phosphorylates proteins, thus producing physiologic effects. Stimulatory ligands such as ACTH, bind to the stimulatory receptor (R_S) and activate C via G_S, the stimulatory nucleotide regulatory protein. Inhibitory ligands inhibit C via the inhibitory receptor (R_i) and the inhibitory G protein (G_i).

Angiotensin II is subsequently cleaved to angiotensin III, which can also increase aldosterone secretion. The stimulatory effect of angiotensin II and angiotensin III on aldosterone secretion result in the inhibition of renin release from the kidney and aldosterone secretion from the adrenal gland. This important negative feedback mechanism which is involved in the regulation of aldosterone, sodium homeostasis and blood pressure is illustrated in figure 1.7.

Angiotensin II stimulates aldosterone secretion by binding to specific high-affinity receptors located on the plasma membrane of the zona glomerulosa cell, a reaction which can be blocked by angiotensin antagonists such as saralasin (Goodfriend *et al* 1970). The angiotensin II receptors are thought to be coupled to at least 2 guanine-nucleotide binding proteins (G-proteins), which act as transducers of the primary interaction with the receptor and the initiation of the intracellular events leading to cellular response (Hausdorff *et al* 1987). One of these proteins is known to be the inhibitory G-protein (G_i),which plays no role in the stimulatory action of angiotensin II, but does mediate the inhibitory effects of high concentrations of angiotensin II on aldosterone secretion. The second G-protein does mediate the stimulatory effect of angiotensin II on aldosterone production but has yet to be fully characterised. However, it is insensitive to pertussis toxin, indicating that it is not the common stimulatory G-protein (G_S) linked to a number of other receptors such as ACTH. (Figure 1.6b)

Aldosterone production in response to angiotensin II is increased by a mechanism which does not appear to involve the coupling of the G-proteins to adenylate cyclase, the enzyme which catalyses production of the common second messenger, cyclic AMP. This remains true even in the presence of theophylline, an inhibitor of phosphodiesterase, the enzyme responsible for cyclic AMP breakdown (Tait *et al* 1974, Shima *et al* 1978). Indeed some studies have shown cyclic AMP levels to actually decrease. However, this was later attributed to either an impure cell preparation or increased phosphodiesterase activity, probably caused by the G_i protein (Albano *et al* 1974, Shima *et al* 1978, Fakunding *et al*

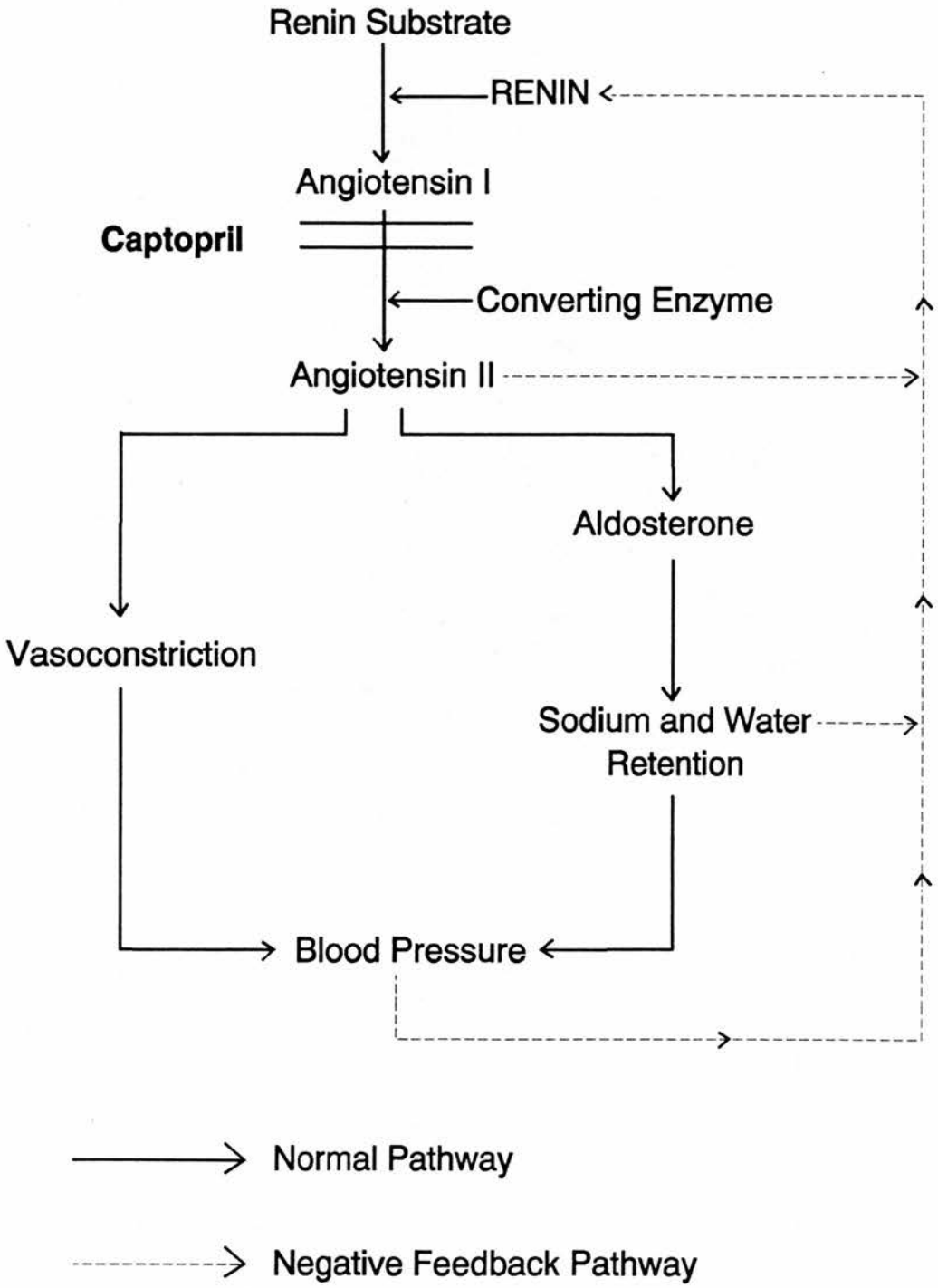


Figure 1.7 **The renin angiotensin system and the negative feedback system**

1979). Therefore, with no apparent role for cyclic AMP, attention has focussed on the role of calcium (Ca^{2+}) as a second messenger, and it is now clear that changes in intracellular Ca^{2+} concentration coupled to changes in phosphatidylinositol metabolism are key events in the mechanism of action of angiotensin II.

Ca^{2+} is particularly suited to act as a second messenger because of the very low (10^{-7} M) concentration of intracellular ionised Ca^{2+} compared with the much higher extracellular level (10^{-3} M). This equilibrium is maintained by the cell membrane which at rest is highly impermeable to Ca^{2+}, by a Ca^{2+} pump which expels Ca^{2+} at the expense of ATP and also by intracellular proteins and organelles which have a high affinity for the Ca^{2+} ion. Consequently, any increase in intracellular concentration caused by a particular stimulus is detected by the cell as a signal or message.

The involvement of extracellular Ca^{2+} in the action of angiotensin II was shown by many groups, who demonstrated in isolated cells that removal of extracellular Ca^{2+} from the incubation medium using chelating agents, or prevention of Ca^{2+} transport across the plasma membrane using Ca^{2+} antagonists such as lanthanum, verapamil and tetracaine, completely abolished steroidogenesis (Shima *et al* 1978). However, unlike ACTH, the binding of angiotensin II to its receptor was unaffected (Fakunding *et al* 1979). Radiolabelled $^{45}Ca^{2+}$ influx studies have demonstrated both Ca^{2+} influx and efflux. Direct experiments using highly purified glomerulosa cells loaded with Quin-2 or Fura-2, specific dyes which fluoresce intensely on binding Ca^{2+}, have shown a clear biphasic increase in the concentration of cytosolic Ca^{2+} following stimulation by angiotensin II. Firstly, a rapid transient increase, leading to a secondary but sustained increase. The sustained rise is thought to be of extracellular origin as it can be blocked by Ca^{2+} antagonists, whilst the primary transient increase is derived from a dantrolene-sensitive non-mitochondrial intracellular pool, most probably the ER (Foster and Lobo 1981, Williams *et al* 1981, Foster and Rasmussen 1983, Capponi *et al* 1984, Braley *et al* 1986, Kojima *et al* 1985a). Following its translocation to the cell cytosol, Ca^{2+} can mediate the cellular response by binding to specific proteins which

require Ca^{2+} for activity. One of these proteins is calmodulin, a ubiquitous compound first described by Cheung in 1982. Calmodulin itself is devoid of enzymatic activity. However, on binding Ca^{2+} it undergoes conformational changes which alter its association with other cellular enzymes such as phosphorylase kinase and calmodulin dependent protein kinases I, II and III, rendering them active and capable of phosphorylating a number of other compounds which eventually initiate the cellular response. Calmodulin has also been shown to activate phosphodiesterase which may account for the fall in cyclic AMP levels following angiotensin II stimulation observed by Bell *et al* 1981.

Closely coupled to the ionic events is the phosphatidylinositol (PI) system. Following binding of angiotensin II to its receptor, and transduction of the initial signal by the G-proteins, phosphatidylinositol 4, 5-bisphosphate (PIP_2) is hydrolysed by the enzyme phospholipase C leading to the rapid formation of inositol 1,4,5-triphosphate (IP_3) and Sn-1,2,-diacyl glycerol (DAG) (Farese 1984a, Farese *et al* 1984b, Enyedi *et al* 1985). The IP_3 mobilises Ca^{2+} from the intracellular pool, whilst DAG is a known activator of protein kinase C. Protein kinase C causes phosphorylation of the proteins ultimately responsible for stimulating the steroidogenic response, and it can be activated by using phorbol esters such as 12-O-tetradecanoyl phorbol-13-acetate (TPA). Although discussed separately, the Ca^{2+} and PI system are very closely integrated and the activation of both systems is required for maximal steroidogenesis. This was clearly shown using the Ca^{2+} ionophore A23187 and TPA. In an isolated cell system both drugs were required in the incubation to induce maximal steroidogenesis (Kojima *et al* 1983, 1984).

(Figure 1.7b)

The synergistic effects of the Ca^{2+} and PI second messenger system on the steroidogenic response are a clear example of a cellular cascade mechanism, whereby a small signal is transposed into a large response via a number of intracellular events.

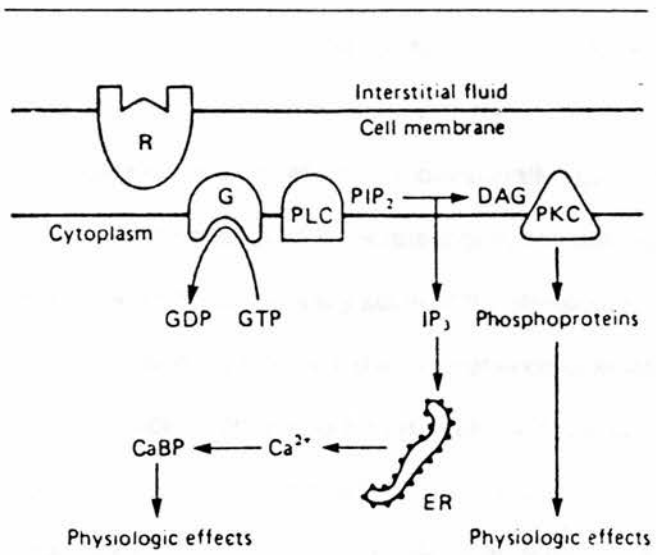


Figure 1.7b. The phosphatidylinositol second messenger system. Binding of ligand e.g. angiotensin II, to receptor (R) activates phospholipase C (PLC) via a G protein (G_p). The resulting hydrolysis of PIP_2 produces IP_3, which releases calcium from the endoplasmic reticulum (ER) and DAG, which activates protein kinase C (PKC). Binding of calcium to calcium binding proteins (CaBP) and the phosphorylation of proteins by PKC leads to the initiation of physiologic effects.

1.7.2. ACTH

A number of pituitary-derived factors increase aldosterone secretion, including pro-opiomelanocortin (POMC), γ-melanocyte stimulating hormone (γ-MSH), α-MSH, ß-MSH, β–lipotropic hormone (β-LPH) and ß-endorphins (Vinson *et al* 1980, Matsuoka *et al* 1981, Seidah *et al* 1981). However, ACTH, which is more usually concerned with the control of glucocorticoid secretion, is the most potent secretagogue, stimulating aldosterone secretion both *in vitro* and *in vivo*, by acting on the early stage of the steroidogenic pathway (Davis *et al* 1975, Fraser *et al* 1979). However, its role in the maintenance of aldosterone secretion under normal physiological conditions appears to be an acute one, as either hypophysectomy or dexamethasone suppression have no effect on circulating levels of aldosterone (Himathongkam *et al* 1975, Fraser *et al* 1979). Sodium depletion and potassium loading enhance the aldosterone response to ACTH, whereas chronic ACTH deficiency reduces the response to sodium depletion (Kem *et al* 1975, Davis *et al* 1975, Fraser *et al* 1979, Coghlan *et al* 1979).

ACTH binds to specific cell surface receptors. Two populations of binding site have been reported, a low affinity-high capacity site and a high affinity-low capacity site, the latter of which is well correlated to the concentration of ACTH required to produce half maximal stimulation of aldosterone secretion (Gallo-Payet *et al* 1985). Binding of ACTH to its receptor causes activation of the membrane bound adenylate cyclase, resulting in generation of the second messenger cyclic AMP (Kojima *et al* 1985b). The ACTH receptor is coupled to adenylate cyclase by a stimulatory G protein (G_S) which binds GTP and stimulates the enzyme and cyclic AMP production respectively (Kojima *et al* 1985b). The cyclic AMP generated binds to inactive cyclic AMP-dependent protein kinases which consist of two distinct sub-units; one of a catalytic nature, the other regulatory. Cyclic AMP binds to the regulatory sub-unit, causing dissociation and subsequent release of the active catalytic moiety. This active unit catalyses the phosphorylation of specific proteins which mediate the steroidogenic response. There appear to be a number of cyclic AMP-dependent protein kinases, which

integrate to phosphorylate common protein substrates. Meanwhile the G_S-protein slowly hydrolyses guanosine tri-phosphate (GTP) to guanosine di-phosphate (GDP) and phosphate (P), inactivating the adenylate cyclase moiety, and thus terminating cyclic AMP production and the end response.

Some studies have suggested that different signal transduction mechanisms operate, depending on the concentration of ACTH, for example at concentrations $>10^{-9}$ M, cyclic AMP and steroid output are increased, whereas at $< 10^{-10}$ M there is no increase in cyclic AMP although steroidogenesis is enhanced (Kojima *et al* 1985b).

In the action of ACTH, Ca^{2+} again plays a key role in the coupling of the initial signal to the end response and it is closely associated with activation of the adenylate cyclase system. The requirement of extracellular Ca^{2+} was shown by a number of groups who demonstrated that in the absence of extracellular Ca^{2+}, cyclic AMP and aldosterone secretion were inhibited, and ACTH had a greater effect on cyclic AMP production when extracellular Ca^{2+} was elevated (Fakunding *et al* 1979, Shima *et al* 1979). The former effect was later partly attributed to the requirement of Ca^{2+} for ACTH binding to its receptor (Cheitlin *et al* 1985). However, Ca^{2+} is also required at several stages in the transduction process subsequent to receptor binding. Stimulation of the transmembrane influx of Ca^{2+} by ACTH was demonstrated by $^{45}Ca^{2+}$ studies, however, Ca^{2+} efflux was unaffected (Leier and Jungman 1973, Williams *et al* 1981, Kojima *et al* 1985b). Further studies showed similarly that Ca^{2+} was not mobilised from an intracellular pool as was found with angiotensin II, but was derived solely from an extracellular source (Kojima *et al* 1985b). Ca^{2+} itself can partially mimic the steroidogenic effect of ACTH, although A23187, a Ca^{2+} ionophore, only weakly stimulates secretion, indicating a permissive rather than mediatory effect of Ca^{2+} on steroidogenesis.

The events which couple Ca^{2+} influx to the cellular response are similar to those induced by the action of angiotensin II, i.e. binding of the Ca^{2+} to calmodulin resulting in the activation of protein kinases and phosphodiesterase which result in increased steroidogenesis and the termination of the cyclic AMP response respectively.

1.7.3. Sodium

In 1954 Leutscher and Axelrad first observed a link between sodium status and aldosterone secretion, and it is now apparent that sodium depletion increases both aldosterone secretion and the width of the zona glomerulosa. Conversely, sodium loading decreases aldosterone secretion and zona glomerulosa width. However, the exact mechanism of this has remained somewhat unclear, mainly due to the large number of physiological effects initiated by altered sodium status whose individual relevance and relative contribution to the change in aldosterone output are poorly understood.

During a low sodium diet, for example, these effects include;

1. Modulation of the renin-angiotensin system, in that lowering sodium increases renin secretion from the juxtaglomerular cells of the kidney, which acts as described in the previous section. An increase in the number of angiotensin II receptors, and subsequent zona glomerulosa resposiveness to angiotensin II has also been observed.
2. Decreased extracellular fluid volume, circulating plasma volume and arterial blood pressure, which may also activate the renin-angiotensin system.
3. Retention of potassium ions, which can stimulate aldosterone production directly.

The effects of sodium on aldosterone secretion therefore appear to be largely indirect, and it has been suggested that its principal action is on the renin-angiotensin system. However, studies in nephrectomised animals whose renin-angiotensin system is therefore absent, have shown that aldosterone output is still enhanced during sodium restriction. Some groups have suggested the involvement of a pituitary-derived substance, not ACTH, which is secreted during sodium depletion and stimulates aldosterone production (McCaa *et al* 1974, Pratt *et al* 1981). Other studies have suggested a direct adrenal action of low sodium, acting at both the early and late stage of the biosynthetic pathway (Davis *et al* 1968, Tait *et al* 1970). More recently a local renin-angiotensin system has been identified in the adrenal cortex which is activated during sodium depletion. This could perhaps account to some extent for the increase in aldosterone secretion during sodium depletion in

nephrectomised animals (Doi *et al* 1983, 1984).

1.7.4. Potassium

In contrast to sodium, potassium loading increases aldosterone secretion whilst potassium depletion decreases it. Acutely, it appears to act exclusively and directly on the zona glomerulosa to stimulate the early step in the aldosterone biosynthetic pathway. However, chronic potassium loading stimulates the conversion of corticosterone to aldosterone, and increases the width of the zona glomerulosa. Chronic potassium depletion can also act directly at the kidney to increase renin release (Abbrecht and Vander 1970, Brunner *et al* 1970).

The mechanism by which potassium stimulates aldosterone secretion has been investigated in the same way as the other stimuli. The aldosterone response, which appears to be maximal at approximately 8.4 mM potassium, can be completely inhibited by the Ca^{2+} channel blocker nifedipine, thus indicating a role for extracellular Ca^{2+} and a voltage-dependent Ca^{2+} channel. $^{45}Ca^{2+}$ influx and efflux studies confirmed that the Ca^{2+} is of extracellular origin and is not mobilised from an intracellular source (Fakunding *et al* 1979 and Williams *et al* 1981). However, some studies using 8-(diethylamino) octyl 3,4,5-trimethoxybenzoate (TMB-8), which blocks release of Ca^{2+} from an internal source, have suggested the involvement of the intracellular pool, though the non-specific and side effects of these agents have yet to be fully characterised (Mackie *et al* 1978, Braley *et al* 1986). Direct experiments using the fluorescent dyes Quin-2 and Fura-2 have shown that the increase in cytosolic calcium could be completely blocked with nifedipine, indicating that the elevated intracellular Ca^{2+} concentration is derived from a purely extracellular source and the response is therefore sustained and not biphasic like that of angiotensin II (Capponi *et al* 1984) .

It is therefore now generally believed that the stimulatory effect of potassium on aldosterone secretion is not a receptor mediated event but results from depolarisation of the

plasma membrane by potassium which causes Ca^{2+} influx via the voltage dependent Ca^{2+} channel. In addition to its effects on Ca^{2+}, several groups have shown that potassium causes a small but significant increase in cyclic AMP production, although this has been disputed by others and shown to be prevalent only at higher concentrations of potassium (Boyd *et al* 1973, Albano *et al* 1974, Tait *et al* 1974, Fakunding *et al* 1979, Kojima *et al* 1985c, Hyatt *et al* 1986). However, it is still unclear if the increase in cyclic AMP is a direct effect of the potassium or indeed secondary to the changes in intracellular Ca^{2+} concentration. No increase in radiolabelled IP_3 or ^3H-inositol incorporation was observed in the presence of increasing potassium concentration, although a small transient decrease in PIP_2 and increase in IP_3 has been reported in isolated cells in the presence of 8.7 mM potassium (Whitely *et al* 1984, Kojima *et al* 1985c, Underwood *et al* 1987).

1.7.5. Serotonin

The stimulatory capacity of serotonin (5-hydroxytryptamine; 5HT) on adrenal steroid secretion was first observed by several groups, who demonstrated that serotonin increased secretion of a compound from isolated adrenal tissue of many species (Rosencrantz 1959, Rosencrantz and Laferte 1960, Connors and Rosencrantz 1962). This compound was not a metabolite of serotonin but a steroid which possessed similar chromatographic properties to aldosterone and caused marked sodium retention when administered to adrenalectomised rats. The compound was therefore tentatively identified as aldosterone. At around the same time it was also shown that rat serum, but not plasma, stimulated corticosteroid production in rat glomerulosa cells, suggesting that a component of the blood clotting system may contribute to the steroidogenic effect (Bakker and Wied 1960). However, they dismissed the role of serotonin as the effect could not be blocked by dihydroergotamine. Some years later it was shown that both human and rat serum caused the same stimulatory effect on steroid secretion and further studies revealed that this could be blocked by the serotonin antagonist

methysergide (Muller and Ziegler 1968, Mendelsohn and Kachel 1981).

Since these initial studies many groups have clearly demonstrated the aldosterone stimulatory properties of serotonin in isolated glomerulosa cells, where it is thought to act like many other of the stimuli at the early stage of the biosynthetic pathway, although the effect does appear to be species dependent (Blair-West *et al* 1962, Haning *et al* 1970, Tait *et al* 1972, McDougall *et al* 1976, Bing and Schulster 1977, Fujita *et al* 1979). Serotonin has no effect on zona fasciculata cells (Müller *et al* 1970, Haning *et al* 1970). *In vivo* studies have proved considerably more difficult to carry out, mainly due to the immediate uptake of serotonin by the platelets or its rapid metabolism to 5-hydroxyindoleacetic-acid (5HIAA). Despite this, stimulatory effects on aldosterone have been reported by a number of groups and others have shown an effect with administration of the precursors to serotonin; 5-hydroxytryptophan (5HTP) and tryptophan (Modlinger *et al* 1979, Al-Dujaili *et al* 1980, Mantero *et al* 1982, Shenker *et al* 1985a,1985b, Maestri *et al* 1988).

The mechanism by which serotonin stimulates steroidogenesis is not entirely clear. Some studies have shown that serotonin can stimulate release of corticotrophin releasing factor (CRF) from rat hypothalamic tissue (Fuller and Clemens 1981, Holmes *et al* 1982). Similarly, it was observed that ACTH and cortisol output, in addition to aldosterone, were also increased when tryptophan or 5HTP was administered to humans (Modlinger *et al* 1979, Maestri *et al* 1988). These results indicate that *in vivo*, serotonin may act centrally to release CRF and subsequently ACTH which stimulates aldosterone secretion. A similar hypothesis was also suggested by Shenker *et al* 1985b, who showed that peripheral blockade of serotonin synthesis using carbidopa, augmented the aldosterone response to 5HTP administration, whilst the serotonin antagonists ketanserin or methysergide had no effect. A centrally mediated action may account for the mechanism of action of serotonin *in vivo*, but they fail to explain the stimulatory effect on isolated cells, which are obviously devoid of any central involvement. They also fail to explain the stimulatory effect of serotonin on perfusion flow and steroid secretion in the isolated perfused rat adrenal gland *in situ* (Hinson *et al*

1989).

A direct action of serotonin on the zona glomerulosa remains a possible theory, however specific receptors for serotonin in the adrenal have yet to be identified and fully characterised as they have been in the CNS, vasculature and platelets (see chapter 2). If these receptors exist then a signal-transduction mechanism would be in operation, and many groups have reported an increase in cyclic AMP output in isolated glomerulosa cells stimulated with serotonin (Albano *et al* 1974, Fujita *et al* 1979). Indirect studies using various Ca^{2+} antagonists, which block the aldosterone stimulating properties of serotonin have also indicated a possible role for Ca^{2+} influx (Ganguly and Hampton 1985). No change in Ca^{2+} efflux from an intracellular source has been observed in isolated zona glomerulosa cells loaded with $^{45}Ca^{2+}$ and stimulated with serotonin suggesting overall that it may act in a similar fashion to ACTH (Williams *et al* 1981). Similarly, studies in isolated zona glomerulosa cells using Ca^{2+} sensitive fluorescent dyes have shown no increase in intracellular Ca^{2+} levels. However, these experiments were carried out in bovine derived cells, which do not respond steroidogenically to serotoninergic stimulation (Capponi *et al* 1987).

The biochemistry of serotonin is discussed more fully in the following chapter and it is the aim of the subsequent chapters of this thesis to answer the unsolved questions pertaining to serotonin and the adrenal gland, and establish its physiological role in the control of aldosterone secretion, which has so far remained elusive.

1.7.6. Aldosterone Stimulating Factor

A protein fraction isolated from human urine was found to increase aldosterone production in isolated zona glomerulosa cells from many species, and also cause hypertension and elevated plasma aldosterone levels when chronically administered to rats (Sen *et al* 1977, Bravo *et al* 1980, Saito *et al* 1981 and Sen *et al* 1981a, 1981b). The compound, termed aldosterone stimulating factor (ASF), was found to be a glycoprotein localised exclusively in the cells of the pituitary anterior lobe and found to be elevated in

patients with idiopathic hyperaldosteronism (Sen *et al* 1981b, Carey *et al* 1984). The stimulatory properties of ASF, unlike angiotensin II, remain unaffected by sodium depletion or by the introducton of antagonists of angiotensin II or ACTH, indicating that the stimulatory properties of ASF could not be attributed to an interaction with the angiotensin II or ACTH receptor. High concentrations of ASF do however potentiate the aldosterone response to angiotensin II in isolated zona glomerulosa cells, but only during sodium depletion. Conversely, similar concentrations of ASF inhibit the response to ACTH in both sodium deplete and replete conditions (Hata *et al* 1984, Bravo *et al* 1980, Saito *et al* 1981).

1.7.7. Catecholamines

Catecholamines, e.g adrenaline and noradrenaline, are stored in the chromaffin cells of the adrenal medulla and are normally concerned with control of glucocorticoid biosynthesis in the zona fasciculata, a reaction facilitated by the close proximity of the two areas. However, De Lean *et al* 1984a, showed that both noradrenaline and analogues of noradrenaline could stimulate aldosterone production *in vitro* with a concommitant increase in cyclic AMP. This process is mediated by ß-1 receptors.

1.7.8. Vasopressin

Arginine vasopressin (AVP) has been shown to increase aldosterone secretion *in vitro* in a cell superfusion system though the effect is small and transient (Balla *et al* 1985). No stimulatory effects have been reported in either a static cell system or *in vivo* (Vinson *et al* 1981). However, at low concentrations it potentiates the aldosterone response to low doses of ACTH. Conversely, at higher concentrations AVP blocks the response to high doses of ACTH (Payet and Lehoux 1982).

Specific V-2 receptors have been identified on zona glomerulosa cells, and these are thought to be coupled to the PI second messenger system as PIP_2 breakdown and IP_3 formation has been demonstrated (Woodcock *et al* 1986). In addition, AVP has been

localised in the adrenal medulla using RIA, HPLC and histochemical techniques (Ang and Jenkins 1984). This is thought to influence the secretion of catecholamines which, as discussed previously, can stimulate corticosteroid secretion.

1.7.9. Prostaglandins

Prostaglandins were first implicated in the control of aldosterone secretion by Flack *et al* 1969, who showed a stimulatory effect on aldosterone output *in vitro* by prostaglandins $F_{1\alpha}$, E_1 and E_2. However, conflicting results were reported by Spät and Jozan 1975, who showed a stimulatory effect by prostaglandins only of the type E.

It has been suggested that prostaglandins may play a role in the enhanced aldosterone response to ACTH, observed during sodium depletion, as indomethacin, an inhibitor of prostaglandin synthesis, blocks the enhanced response (Morise *et al* 1982).

1.7.10. Atrial natriuretic peptide

In 1981 de Bold *et al* demonstrated that intravenous administration of atrial extracts to rats provoked a slight fall in arterial blood pressure and a large natriuresis, which was absent when similar experiments were conducted using ventricular tissue. The compound(s) responsible for these effects were found in atrial granules similar to those of the kidney JG cells and were termed the atrial natriuretic peptides (ANP). It was found that the granularity could be altered by water and salt depletion and adrenalectomy (Kisch 1956, Jamieson and Palade 1964, de Bold *et al* 1978)

Since these early studies a number of groups have since purified and fully characterised the various peptides in humans and other species (Thibault *et al* 1983, Atlas *et al* 1984). Consequently, synthetic ANP has been manufactured and used in a number of experiments, where it has been shown to inhibit basal aldosterone secretion both *in vivo* and *in vitro*, in addition to the aldosterone response to angiotensin II, potassium and ACTH

(Chartier *et al* 1984, Maack *et al* 1984, Atarashi *et al* 1984, Volpe *et al* 1985). More recently, work by Espiner and Richardson 1989, has shown that infusion of physiological doses of ANP decreases aldosterone secretion, PRA and systolic blood pressure.

The mechanism by which ANP decreases aldosterone production is not entirely clear. Specific membrane receptors of both a high and low affinity have been identified, which are distinct from those of angiotensin II and ACTH (De Lean *et al* 1984b,1984c). ANP has been shown to inhibit adenylate cyclase, and although this may account for the inhibitory effect on the action of ACTH, it fails to explain the effect on angiotensin II stimulated aldosterone output, which does not involve adenylate cyclase (Anand-Srivastava *et al* 1984). In addition to its effects on adenylate cyclase, ANP has been shown to increase cyclic GMP production. However, it is unclear which is the primary mechanism (Waldman *et al* 1984). It has also been postulated that ANP may act as a Ca^{2+} channel blocker, and this may explain its inhibitory effect on angiotensin II, potassium and ACTH stimulated aldosterone secretion, which all utilise calcium as a second messenger. However, the effect of ANP on Ca^{2+} entry is controversial, with some groups reporting inhibition and others reporting no change. (Capponi *et al* 1986, Chartier and Schiffrin 1987). However, recently patch clamp techniques have shown that in bovine cells, ANP inhibits T(transient) -type Ca^{2+} channels and stimulates L(long lasting, large capacitance)-type channels (McCarthy *et al* 1990).

In addition to regulating steroid biosynthesis, ANP may also act as an adrenal growth factor as it stimulates incorporation of thymidine into the DNA of adrenal zona glomerulosa cells in primary culture (Horiba *et al* 1985).

1.7.11. Dopamine

A possible role for dopamine in regulating aldosterone secretion came largely from pharmacological *in vivo* experiments using the dopamine antagonist metoclopramide which enhanced aldosterone secretion but not plasma renin activity (PRA), ACTH or plasma potassium , indicating that under normal circumstances aldosterone secretion is under tonic

inhibition by dopamine (Carey *et al* 1979). Although this theory is not supported by other workers who have suggested that the inhibition of aldosterone synthesis by dopamine is an extra-adrenal effect caused by increased renal clearance of angiotensin II (Inglis *et al* 1987, Connell *et al* 1987). Further studies, using a rat adrenal cell superfusion system, showed that the steroid enhancing effects of metoclopramide could be blocked by dopamine, but not angiotensin II (Brown *et al* 1977, Carey *et al* 1979, Edwards *et al* 1980a). However, Aguilera and Catt 1984, demonstrated that during sodium repletion the sensitivity of the aldosterone response to angiotensin II was enhanced in the presence of metoclopramide, suggesting that the antagonist reversed the inhibitory effect of dopamine. However, in sharp contrast, other groups have failed to demonstrate a stimulatory effect of metoclopramide on aldosterone secretion either *in vivo* or *in vitro* or a inhibitory effect of dopamine itself on aldosterone secretion, (Campbell *et al* 1981, Hampton and Ganguly 1986) and also the serotoninergic agonist properties of metoclopramide must be remembered, which makes interpretation of these results difficult. *In vitro* studies using isolated bovine and rat zona glomerulosa cells with dopamine itself are less conclusive. These have shown that dopamine has either no effect or only a moderate inhibitory effect on basal aldosterone secretion or secretion stimulated with angiotensin II or ACTH and only at very high concentrations of $> 10^{-5}$ M (Boscaro *et al* 1979, Edwards *et al* 1980a, McKenna *et al* 1979, Aguilera and Catt 1984, Sequeira and McKenna 1985). In contrast, infusions of angiotensin II plus dopamine in sodium deplete human impeded the increased sensitivity to angiotensin II normally observed. This regulatory role of dopamine during altered sodium status has been supported by other studies which have shown a decrease in urinary dopamine during sodium depletion and conversely an increase during sodium loading (Alexander *et al* 1974, Carey *et al* 1981).

These effects on the adrenal are thought to be mediated by specific dopamine receptors located on the zona glomerulosa. Recently these receptors have been classified into DA-1 and DA-2 sub-types. The mechanism of dopaminergic action is not entirely clear, though some groups have suggested that the DA-1 receptor stimulates adenylate cyclase

activity and aldosterone production, whilst the DA-2 receptor inhibits adenylate cyclase and aldosterone (Missale *et al* 1986).

The dopamine involved in the control of aldosterone secretion may be derived from the circulation or the adrenal itself as several groups have shown the presence of dopamine containing chromaffin granules in many species. Further support for the local effect are studies which showed that the stimulatory effects of metoclopramide infusion on aldosterone secretion was not altered by ganglionic blockade of the autonomic nervous system (Van Loom *et al* 1980, Wilson *et al* 1983). The dopaminergic mechanism appears to be independent of the nervous system as adrenal catecholamine content is not depleted by splanchnic nerve section or central inhibitors of catecholamine biosynthesis (McCarty *et al* 1984).

1.7.12. Histamine

The role of histamine in the control of aldosterone secretion was first shown by Edwards *et al* 1980b, using isolated superfused rat adrenal zona glomerulosa cells. The H_2 antagonists cimetidine and ranitidine elevated basal aldosterone secretion and blocked the response to angiotensin II, but not serotonin, ACTH or potassium. In contrast, the H_1 antagonist chlorphenhydramine, enhanced the aldosterone response to both angiotensin II and ACTH, indicating a possible link between the histamine and the angiotensin II receptor.

1.7.13. Somatostatin

Somatostatin inhibits the aldosterone response to angiotensin II, but not ACTH, potassium or 8-bromo-cyclic AMP (Aguilera *et al* 1981a). However, at high concentrations it moderately increases basal aldosterone secretion in isolated zona glomerulosa cells. This agonistic effect has been attributed to a weak interaction of the peptide with the angiotensin II receptor. Firstly, as it can be blocked by the addition of saralasin and secondly, in high

concentrations somatostatin can displace radiolabelled angiotensin II from its binding site. In contrast, the inhibitory effect of somatostatin on angiotensin II stimulated aldosterone output is probably due to an interaction with its own specific receptor within the adrenal. Indeed studies have demonstrated specific binding sites within the adrenal zona glomerulosa and immunohistochemical studies have shown the presence of somatostatin itself, although this has been disputed by other groups (Aguilera *et al* 1981,1982, Srikant and Patel 1985).

1.8. Disorders of mineralocorticoid secretion

1.8.1. Mineralocorticoid excess

Overproduction of aldosterone associated with suppressed renin is termed primary or idiopathic hyperaldosteronism, or more commonly Conn's Syndrome. It is caused by either an adrenal adenoma, micro or macro nodular hyperplasia or more rarely a carcinoma. The symptoms of this disease are hypertension, headaches and hypokalaemia leading to muscle weakness and polyuria. Oedema rarely occurs. Diagnosis is based on elevated plasma aldosterone and low renin levels. If only one gland is affected, treatment is by unilateral adrenalectomy. If both adrenals are involved, large doses of an aldosterone antagonist, such as spironolactone which is now known to have potentially dangerous side effects, or amiloride, are administered.

Secondary hyperaldosteronism results from over production of renin, causing a subsequent increase in angiotensin II and therefore aldosterone, resulting in the same symptoms as the primary condition. The cause of the persistent renin secretion may be due to a tumour of the juxtaglomerular cells of the kidney or hyperplasia (Bartter's Syndrome). A positive diagnosis is made from the plasma levels of aldosterone and renin which are both elevated. Treatment is similar to that of the primary condition.

1.8.2. Mineralocorticoid deficiency

Addison's disease is the result of autoimmune or idiopathic atrophy, causing both

mineralocorticoid and glucocorticoid deficiency. Hypo-secretion of aldosterone results in increased natriuresis, diuresis, plasma potassium concentration, and decreased plasma sodium and blood volume, resulting in postural hypotension and faintness. Glucocorticoid deficiency leads to high levels of plasma ACTH, an attempt by the body to increase steroid secretion from the damaged gland, a condition which also causes skin pigmentation. Patients normally present during an Addisonian crisis i.e abdominal pain, fever and hypotensive collapse. Confirmation of Addison's disease is by an ACTH stimulation test, wherby administration of synthetic ACTH (Synacthen) fails to stimulate cortisol production. The immediate treatment is by intravenous infusion of saline and hydrocortisone and maintenance with glucocorticoid and mineralocorticoid replacement therapy.

Chapter Two

The Biochemistry of Serotonin.

2.1. Introduction

Serotonin is a potent vasoconstricting agent, present in serum after blood has clotted, and with the knowledge that serotonin may therefore affect blood vessels throughout the body, many researchers have focussed their interests on the potential role of serotonin as a humoral agent responsible for the development and maintenance of hypertension and cardiovascular disease. However to date, despite many years of intensive clinical and laboratory studies, the physiological and pathophysiological relevance of serotonin in man remains elusive.

2.2. Discovery of serotonin

In 1933 Erspamer and Asero isolated a phenolic compound called enteramine from the gastric mucosa, which stimulated contraction of intestinal and uterine muscle strips. Some 15 years later Rapport and his colleagues isolated a potent vasoconstrictive compound from serum which they called serotonin. The former group later confirmed in 1952 that enteramine and serotonin were in fact the same compound, systematically named 5-hydroxy-3-(2-aminoethyl)-indole or more simply 5-hydroxytryptamine (5HT) and commonly now known as serotonin (Figure 2.1).

2.3. Biosynthesis of serotonin

With the exception of platelets, serotonin is synthesised in most of the tissues in which it is stored, including the central nervous system (CNS) and the pineal gland. However the enterochromaffin cells of the gut, also known as amine precursor uptake and decarboxylation (APUD) cells remain the major site of biosynthesis in man (Pearse 1968) .

Serotonin is synthesised from the essential amino acid L-tryptophan by two enzymatic reactions (Figure 2.2). These represent a minor metabolic pathway for tryptophan and utilise approximately 2% of the total dietary intake. The first step which converts tryptophan to 5-hydroxytryptophan (5HTP) is catalysed by the enzyme tryptophan hydroxylase. This has

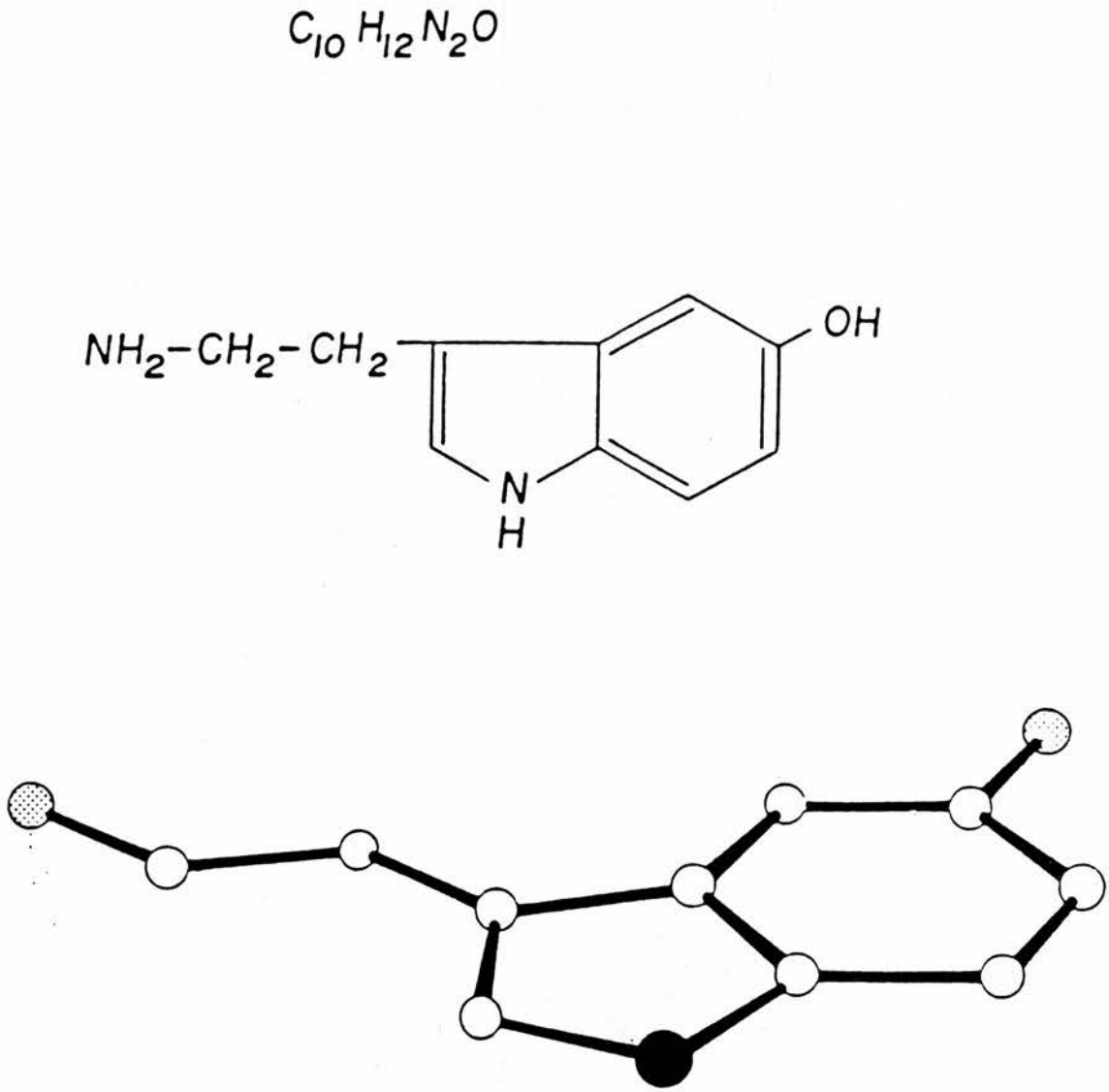


Figure 2.1. **The chemical composition and structure of serotonin.**

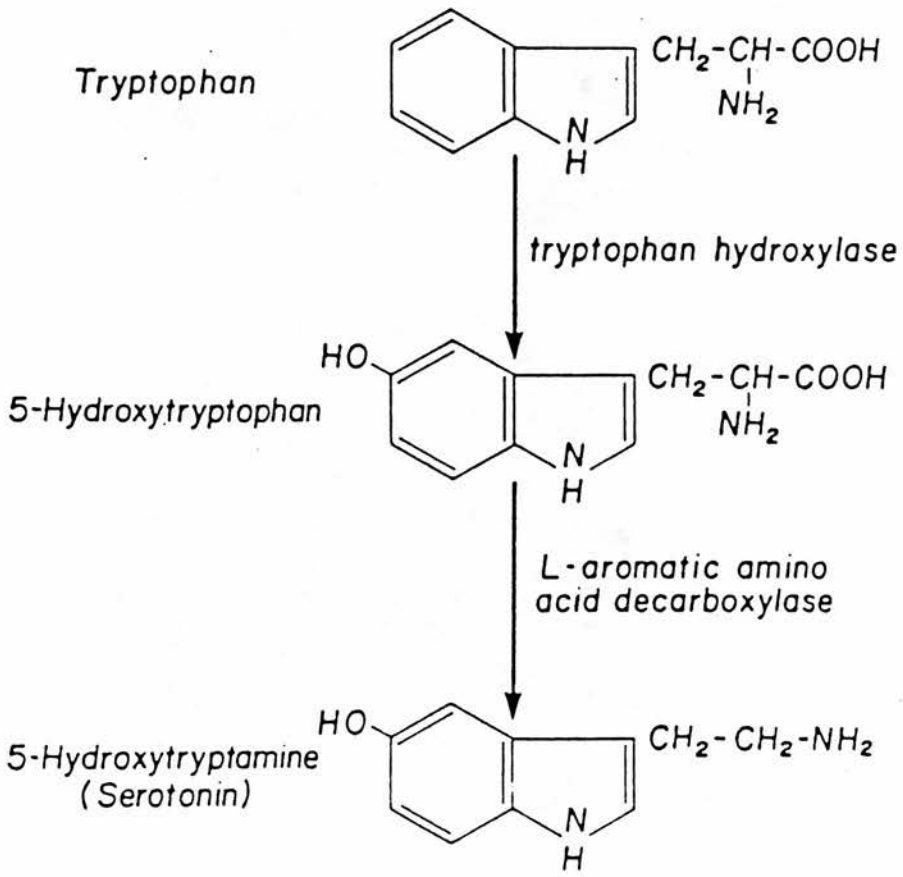


Figure 2.2. **The biosynthesis of serotonin.**

been localised by immunohistochemical staining extensively in the brain and periphery but not in platelets (Joh *et al* 1975, Gershon *et al* 1977a, Morrissey *et al* 1977). The enzyme requires tetrahydrobiopterin as a co-factor and is easily inactivated by molecular oxygen (Kuhn *et al* 1980a). The K_m is approximately 3×10^{-5} M, which is high in comparison with the normal circulating levels of tryptophan (Tong and Kaufman 1975). Therefore, as it is not saturated with substrate its action appears to be the rate limiting step, and thus a point of control in the serotonin biosynthetic pathway. Tryptophan hydroxylase activity can be inhibited both *in vivo* and *in vitro* by the amino acid p-chlorophenylalanine (PCPA), causing a subsequent fall in serotonin concentration, and thus providing a useful tool to investigate the effects of serotonin depletion (Jecquier *et al* 1967).

The second step, which converts 5-hydroxytryptophan to serotonin, is catalysed by L-aromatic amino acid decarboxylase, an enzyme which also catalyses the decarboxylation of many other amino acids e.g tyrosine and phenylalanine to form catecholamines, such as dopamine. It is located in a number of tissues, particularly the liver and kidney and also the adrenal gland. It requires pyridoxal phosphate as a co-factor, which paradoxically, inhibits the enzyme when present in excess i.e > 6×10^{-7} M (Bouchard and Roberge 1979). The K_m for this enzyme is low i.e 8×10^{-6} M and consequently is not a rate limiting step. Inhibition of this step by carbidopa and other amino acid analogs provide yet another useful method of depleting circulating levels of serotonin, however, it must be remembered that a concomitant decrease in the levels of circulating catecholamines will also occur (Gershon *et al* 1977b).

2.4. Metabolism of serotonin

The primary route of serotonin metabolism (Figure 2.3) is by oxidative deamination to the unstable intermediate 5-hydroxyindoleacetaldehyde by monoamine oxidase, a ubiquitous enzyme localised on the outer mitochondrial surface, and requiring FAD+ as a co-factor (Tipton *et al* 1976). This enzyme exists in 2 forms (Johnston 1968). The type

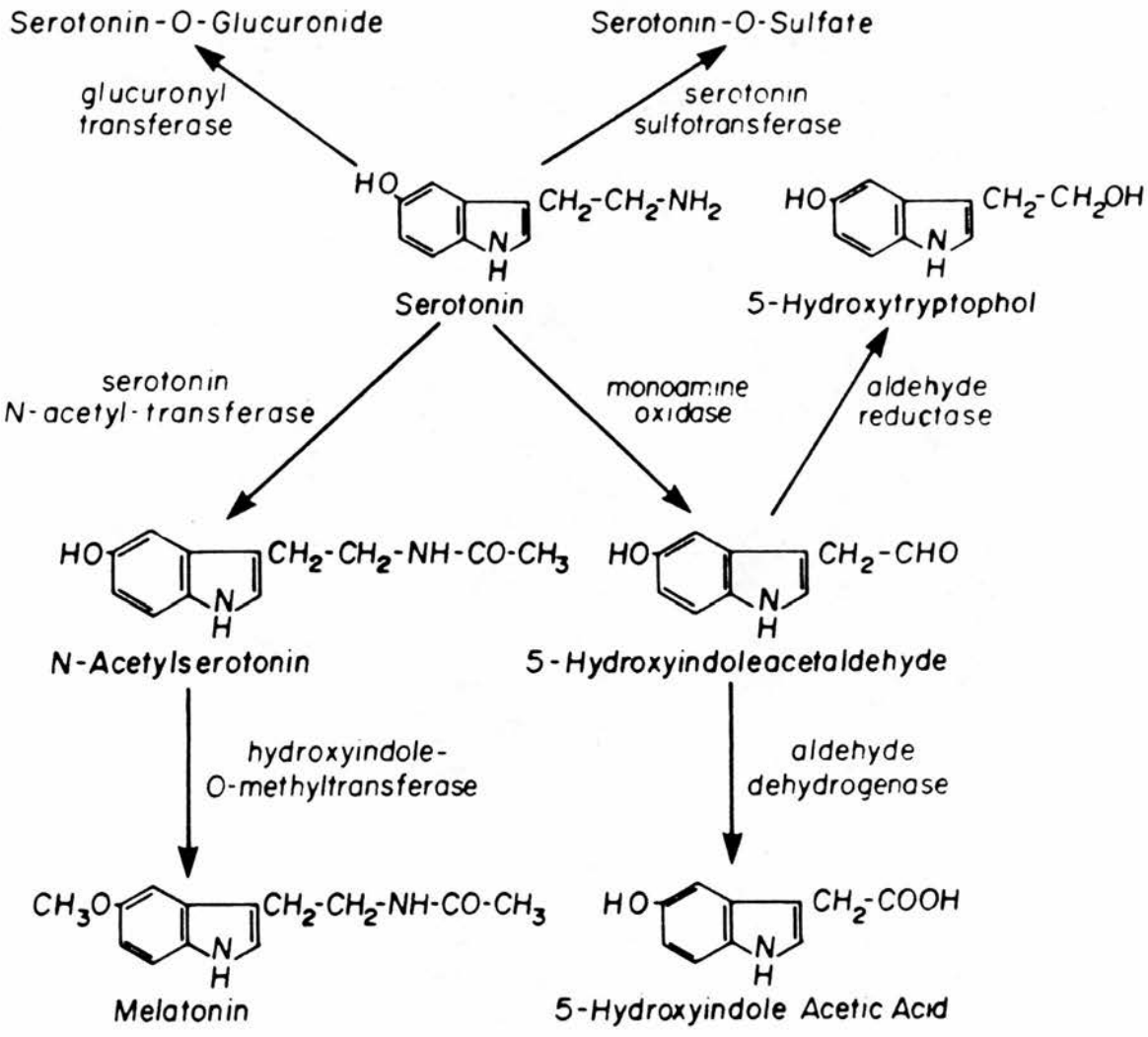


Figure 2.3. The metabolism of serotonin.

A , found both intra and extra-neuronally, is responsible for the deamination of serotonin and noradrenaline, and secondly the type B, solely extra-neural, which deaminates other amines including dopamine and phenylalanine (Youdim and Ashkenazi 1982). Under normal conditions the 5-hydroxyindoleacetaldehyde is rapidly converted to 5-hydroxyindoleacetic acid (5HIAA) by aldehyde dehydrogenase. This is the major urinary excretory product in mammals. Alternatively, and in particular following ingestion of ethyl alcohol, the oxidative pathway is switched off and the reductive pathway, catalysed by aldehyde reductase, is switched on forming 5-hydroxytryptophol (Davis *et al* 1967). This enzyme requires NADP as a co-factor and has a high affinity for its substrate ($K_m = 4 \times 10^{-6}$ M) (Turner and Tipton 1972).

Other minor pathways of serotonin metabolism include sulfation by serotonin-sulfotransferase to form the biologically inactive serotonin-o-sulfate. However, only a very small amount of serotonin-o-sulfate can be detected even when the monoamine oxidase pathway is blocked (Hammond *et al* 1981,1984). This step can be reversed by aryl sulfatase enzymes, indicating that sulfation may therefore serve as a temporary serotonin inactivating system (Kishimoto *et al* 1961).

Glucoronidation, a common detoxifying mechanism which takes place mainly in the liver, is catalysed by uridine diphosphate glucoronyltransferase. This reaction forms serotonin-o-glucoronide which is excreted in large quantities when the monoamine oxidase pathway is blocked (Tyce *et al* 1968).

N-acetylation is the initial step in the conversion of serotonin to the hormone melatonin, formed primarily in the pineal gland. Serotonin is converted to N-acetylserotonin by serotonin-N-acetyltransferase, which is localised within the cell cytosol, and requires acetyl co-enzyme A (Paul *et al* 1974). This is then methylated to form melatonin by hydroxyindole-O-methyltransferase which utilises S-adenosylmethionine as the methyl donor. The activity of serotonin N-acetyltransferase exhibits a marked rhythm throughout the day, being highest at night and lowest during the day. Consequently, the levels of melatonin exhibit a similar diurnal rhythm (Klein and Weller 1970).

2.5. Storage and release of serotonin

Serotonin is widely distributed throughout the plant and animal kingdom, and storage sites have been located in the pituitary gland, the pineal gland, the pancreas and of particular interest to this project, the adrenal gland (Saavedra *et al* 1974, Verhofstadt and Jonsson 1983, Holzwarth *et al* 1984, Koevary *et al* 1983). However, the most common sites of storage are discussed in the sections below.

2.5.1. Serotonin in the central nervous system

Neurones containing serotonin were first identified in the central nervous system using fluorescent histochemical techniques (Dahlstrom and Fuxe 1964). Serotonin is formed locally within the neurones as it does not easily cross the blood-brain barrier. These neurones are easily identified by the presence of tryptophan hydroxylase, the first enzyme in the serotonin biosynthetic pathway (Kuhar *et al* 1972) (see section 2.2). Although serotonin itself does not readily cross the blood-brain barrier, 5HTP the immediate precursor does and systemic administration of this compound increases both cerebral and peripheral levels of serotonin. Similarly depletion of serotonin levels in the CNS can be carried out again using PCPA as previously described or using the cytotoxic agents 5,6 dihydroxytryptamine or 5,7 dihydroxytryptamine which are taken up by the neurons.

Serotoninergic neurones are derived from clusters of serotonin containing cell bodies situated in the raphe nucleii of the brain stem, which project both upwards and downwards to various areas of the CNS. The downward projecting neurones which terminate in the spinal cord are associated with spinal motor reflexes and the conduction of painful stimuli, whilst the ascending neurones project towards the forebrain structure, particularly the cortex, striatum and hippocampus where they are thought to be involved in depression, anxiety and behavioral patterns. A great deal of interest has focussed on the ascending neurones projecting towards the hypothalamus, which itself is known to play a key role in cardiovascular function.

2.5.2. Serotonin in the gastro-intestinal tract

Serotonin is synthesised, as previously described, and stored in the enterochromaffin (APUD) cells of the gut, where it has a short half life of 10-17 hours (Udenfreind and Weissbach 1958, Erspamer and Testini 1959). It is then stored in secretory granules in close association with substance P (Alumets *et al* 1977). Its release into the lumen of the bowel or the portal blood supply from the basolateral membrane of the enterochromaffin cells by emeiocytosis can be triggered by a number of stimuli, including acetylcholine, an increase in transluminal pressure or a decrease in intestinal pH (Forsberg and Miller 1983, Bulbring and Crema 1959, Kellum and Jaffe 1976). Upon its release it is thought to stimulate secretion of pepsin and mucus and inhibit gastric acid production (Menguy 1969, Haverback *et al* 1958). It also increases the motility of the small intestine i.e the peristaltic reflex, in addition to water and electrolyte secretion (Brownlee and Johnson 1963, Kiskoff and Moore 1977).

The alternative source of serotonin in the gut are the serotoninergic neurones which synthesise and take up serotonin by an energy dependent process which requires Na^+ and Ca^{2+} (Gershon and Altman 1971). Release of serotonin from these neurones produces an inhibitory effect on the processes initiated by its release from the enterochromaffin cells.

2.5.3. Serotonin in the blood

The potentially lethal capacity of serotonin to increase vascular permeability and mediate anaphylactic shock means very little, if any, free serotonin is found in the circulatory system of a healthy individual (Majno and Palade 1961, Gershon and Ross 1962). This status is maintained by a number of mechanisms including serotonin binding proteins in the blood e.g serotonectin (Pignatti and Cavalli-Sforza 1975). However, the majority of serotonin in the blood is taken up by platelets. These are small anucleate cells which cannot synthesise serotonin and whose main function is hemostasis. The serotonin is stored within dense granules in close association with cations, nucleotides and proteins, where it has a half life of between 33-48 hours. The serotonin uptake process consists of at least 3 complicated

steps. Firstly, serotonin is transported through the platelet membrane either passively when extracellular serotonin concentration is high i.e > 10 μM or actively when the concentration is less than 5 μM. These processes are aided by serotonectin which is localised on the platelet cell surface. The former passive process is highly temperature dependent, whilst the latter active system depends markedly on the presence of extracellular sodium. Once inside the platelet serotonin is taken up into the membrane enclosed dense granules, a process which again is temperature dependent, but not active, as shown by the apparent ineffectiveness of oubain or iodoacetate as inhibitory agents. Finally, once inside the granules, serotonin forms a complex with ATP and divalent cations, by a mechanism which is poorly understood (Born and Bricknell 1959, Pletscher 1968).

Serotonin is released from the platelets by four main mechanisms; exocytosis, exchange, inhibition of transport at the granular membrane and by aminophores and protonophores. These reactions are triggered by a number of stimuli including thrombin, which is involved in the blood-clotting reaction. The serotonin released acts synergistically with the primary trigger causing further activation and enhancement of the shape change and aggregatory reaction.

Thus platelets play an important role in serotonin storage and it has recently been suggested that they may act as a circulating store of serotonin, which can be released by a process other than irreversible aggregation, at a specific tissue or site which has no nearby source of serotonin but does possess serotonin receptors (Osim and Wylie 1983).

2.6. Serotonin receptors

Initially 2 types of serotonin receptor, both found in the guinea-pig ileum, were described by Gaddum and Picarelli in 1957. One, termed D, which mediated the contraction of enteric smooth muscle could be antagonised by dibenzylamine. The other, termed M, could be antagonised by morphine. This classification was based purely on pharmacological studies. However, with the advent of radioligands, which have proved extremely valuable

research tools, more detailed studies have been made in the classification of the serotonin receptor.

One of the first studies of serotonin receptors using radioligands was carried out in rat brain cortex membranes by Peroutka and Snyder in 1979, who demonstrated that ^{3}H-serotonin and ^{3}H-spiperone, a potent serotonin antagonist, each bound to distinct sites. They named the binding site with high affinity for ^{3}H-serotonin the S_1-serotonergic ($5HT_1$) receptor and that with high affinity for ^{3}H-spiperone the S_2-serotonergic ($5HT_2$) receptor. Recent studies have shown that there is a close correlation between the cortical $5HT_2$ binding site and the previously described D receptor, and that they are in fact probably identical. However, studies with methiothepin and ketanserin, specific antagonists for the $5HT_1$ and $5HT_2$ receptors have shown that the original M receptor cannot be categorised into either the $5HT_1$ or $5HT_2$ receptor types. Therefore a third receptor type clearly exists, which has been termed $5HT_3$. It is now apparent that each of the three receptor types has a specific distribution and function within higher vertebrates and each will be reviewed here briefly. A summary of the characteristics of each of the serotonin receptors is illustrated in table 2.1.

2.6.1. $5HT_1$ Receptors

These receptors bind serotonin in the nanomolar range and have a particularly high affinity for indole derivatives such as ergotamine and metergoline (Peroutka and Snyder 1979). Compared to the $5HT_2$ receptor they exhibit very little affinity for the classical serotonin antagonists such as cyproheptadine. They are located in all areas of the brain, particularly the hippocampus and striatum, and also in the gut where they mediate the inhibitory action of serotonin on acetylcholine release from enteric cholinergic neurones.

Heterogeneity of the $5HT_1$ receptor has been demonstrated by autoradiography and radioligand binding studies, resulting in its sub-classification into the 4 pharmacologically

Table 2.1. **The characteristics of the serotonin receptor sub-types.**

RECEPTOR	$5HT_1$				$5HT_2$	$5HT_3$
SUB-TYPE	a	b	c	d		
LOCATION	Enteric nerves	Cortex	Stomach fundus	Basal Ganglia	Uterus	Vagus
	Dorsal Raphe Nucleus	Sympathetic nerves	Chorold plexus		Platelets	Sensory nerves
		Saphenous vein			Ileum	Sympathetic nerves
	Basilar artery				Brain	Parasympathetic nerves
					Aorta	Enteric nerves
FUNCTION	Neuronal inhibition	Neuronal inhibition	Contraction	?	Contraction	
	Contraction	Contraction			Neuronal depolarisation	Neuronal depolarisation
	Thermoregulation				Platelet aggregation	Transmitter release
	Hypotension					Bezold - Jarisch reflex
SECOND MESSENGER SYSTEM	Modulation of adenylate cyclase	Inhibition of adenylate cyclase	PI Turnover	Inhibition of adenylate cyclase	PI Turnover	?
POTENT PHARMACOLOGICAL AGENTS	8-OH-DPAT	Cyanopindolol	Mesulergine Metergoline Methysergide	Metergoline	Ketanserin Methysergide Mesulergine Spiperone	ICS205/930 Quipazine

distinct $5HT_{1a}$, $5HT_{1b}$, $5HT_{1c}$ and $5HT_{1d}$ sites. The $5HT_{1a}$ site has a high affinity for 8-OH-DPAT and spiroperidol, the $5HT_{1b}$ for cyanopindolol, Ru 24969 and ß-blockers, the $5HT_{1c}$ site binds mesulergine and is analogous to the $5HT_2$ receptor, which also binds mesulergine and the $5HT_{1d}$ site has a high affinity for metergoline (Gozlan *et al* 1983, Hoyer *et al* 1985a, 1985b, Pazos *et al* 1984, Pazos and Palacios 1985).

The second messenger system coupling the $5HT_{1a}$, $5HT_{1b}$ and $5HT_{1d}$ receptors to the end response seems to be primarily by modulation of adenylate cyclase activity. In contrast however, the $5HT_{1c}$ receptor, like the $5HT_2$ receptor, is coupled to the PI second messenger system (Enjalbert *et al* 1978, Peroutka *et al* 1981, Conn and Sanders-Bush 1984, De Chaffoy de Courcelles 1985).

2.6.2. $5HT_2$ Receptors

These receptors bind serotonin and serotonin agonists such as quipazine and bufotenine in the micromolar range, and the specific $5HT_2$ antagonist ketanserin and other antagonists e.g cyproheptadine and methysergide in the nanomolar range (Leysen *et al* 1978). They are found in a number of locations throughout the body, including the cerebral cortex where they mediate neuronal depolarisation, a variety of smooth muscle cells e.g those of the ileum and uterus, where they mediate contraction, and lastly in platelets where two types are thought to exist. Firstly, a low capacity high affinity receptor, involved in aggregation and secondly a high capacity low affinity receptor, antagonised by chlorimipramine and involved in the uptake of serotonin.

In the vascular system serotonin mediates both contraction and dilatation, and it is generally agreed that the contractile response is mediated predominantly by the $5HT_2$ receptor as the effects can be specifically blocked by ketanserin (Cheng *et al* 1980, Peroutka *et al* 1982, Van Neuten *et al* 1981, 1982a, 1982b, 1983). However, other groups have

shown that the contractile properties of 5HT in the rabbit ear artery could be blocked with phentolamine, an α–antagonist, suggesting that in some vascular beds serotonin may interact with α–receptors, and that the anti-hypertensive effect of ketanserin may be due at least in part to its interaction with α_1–receptors (Black *et al* 1981, Fozard 1982).

In addition to its own direct effects serotonin can potentiate the vascular response to other stimuli such as angiotensin II, prostaglandins, histamine and noradrenaline (Van Neuten *et al* 1981, 1982a). These effects can be blocked by ketanserin indicating that the amplification effect of serotonin is mediated by the $5HT_2$ receptor.

The $5HT_2$ receptor is also thought to play an important role in clinical depression, as administration of anti-depressants to rats showed a reduction in $5HT_2$ receptor binding and density (Peroutka and Snyder 1980a, 1980b). Studies in patients using the drug amitryptyline showed similar changes in the $5HT_2$ receptors which correlate well with the therapeutic effect, indicating that depression may in part be due to increased sensitivity of this receptor.

The $5HT_2$ receptors are coupled to phospholipase C and activation results in phosphatidyl inositol breakdown, mobilisation of intracellular calcium and accumulation of diacylglycerol which activates the protein kinase and subsequently the cellular response (De Chaffoy de Courcelles *et al* 1985). A regulatory mechanism has been observed for the $5HT_2$ receptor by Nelson *et al* 1978, who showed that depletion of serotonin using the toxin 5,7, dihydroxytryptamine caused an increase in serotonin binding associated with an increase in receptor density.

2.6.3. $5HT_3$ Receptors

This is the serotonin receptor most recently identified and very little is known about its properties or function. However, these are found on peripheral efferent and afferent

autonomic neurones, and mediate the release of neurotransmitters including noradrenalin from cardiac sympathetic neurones and acetylcholine from cholinergic enteric neurones (Richardson and Engel 1986). Like the $5HT_1$ receptor there appears to be some level of heterogeneity within the $5HT_3$ receptor (Richardson *et al* 1985). The second messenger system to which this receptor is coupled is still unknown, although some data suggests that these receptors are coupled to an ion channel, most probably Ca^{2+} (Neijt *et al* 1988). A number of antagonists bind to this receptor e.g cocaine and ICS 205/930, and it has recently been found that $5HT_3$ antagonists are of considerable therapeutic value, particularly in nausea and vomiting induced by chemotherapy in the treatment of cancer (Fozard 1987).

2.7. The pathophysiological role of serotonin

2.7.1. Carcinoid Syndrome

Serotonin has been loosely associated with a number of clinical disorders, but perhaps the best documented and most positive role is in the Carcinoid Syndrome. In this disease tumourous growth of the enterochromaffin cells of the gut results in the over production of serotonin and consequently its main urinary metabolite 5HIAA, which is used as a marker for the syndrome, and which correlates roughly with the mass of the tumour (Strodel *et al* 1983). The symptoms of the disease are watery diarrhoea, due to inordinate peristalsis and intestinal hypermotility, flushing, abdominal pain and fibrotic disease of the cardiac valves. Treatment is either by surgical removal of the tumour or administration of serotonin antagonists e.g ketanserin, or inhibitors of serotonin synthesis e.g methyldopa, which alleviate the diarrhoea. Anti-histamines are used to treat the flushing (Grahame-Smith 1972).

2.7.2. Hypertension

The etiology of the increase in peripheral vascular resistance in chronic hypertension remains uncertain, although numerous factors including natriuretic peptides, the

renin-angiotensin-aldosterone system and the sympathetic nervous system have all been implicated. Recently however, serotonin has emerged as a possible causative factor.

Serotonin can itself act as a potent vasoconstrictor and vasodilator as well as amplify the pressor effects of other compounds in the periphery. Therefore it would seem logical that elevated serotonin levels may be related to the increased peripheral vascular resistance observed in hypertension. However, abnormal serotonin levels have not been shown in hypertensive patients. Similarly, serotonin antagonists, with the exception of ketanserin, which exhibits affinity for α-adrenergic receptors, do not provoke a large anti-hypertensive effect.

These observations do not totally eliminate a role for serotonin. It may be that the actual vascular response to serotonin is either enhanced or sustained or that the vasodilatory response is suppressed. Evidence for these explanations stem predominantly from studies in spontaneously hypertensive rats (SHRs). Young SHRs have abnormally high levels of noradrenaline and angiotensin II, and although the level of serotonin itself is not increased it acts to augment the pressor effects of these stimuli. Further studies in adult SHRs have shown that the vascular wall is extremely sensitive to the vasoconstrictive properties of serotonin, and tachyphylaxis, the rate of decay of the response to repeated exposure to stimulus, is considerably delayed (Collis and Vanhoutte 1977, 1981). In addition, the vasodilator effect of serotonin is reduced in SHRs, thus causing the vasoconstrictive effect, normally offset by the vasodilatory effect, to be more marked (Kubo and Su 1983). However, in light of this evidence it remains to be shown whether the vascular changes act as a catalyst and provoke hypertension or whether they are in fact secondary to changes resulting from the hypertension itself.

The arrangement of the serotonin containing cell bodies in the CNS closely resemble those involved in cardiovascular regulation, indicating a putative role of serotoninergic neurones in this system. The ascending and descending serotoninergic neurones have been described in a previous section, and their purported role in cardiovascular homeostasis

comes from several studies. Activation of the ascending system by electrical stimulation increases blood pressure and produces variable changes in heart rate (Kuhn *et al* 1980b, 1980c). This response can be attenuated by administration of PCPA, which depletes central serotonin levels, and then partially restored by administration of 5HTP. The pressor response can also be both enhanced and sustained by giving fluoxetine, an inhibitor of serotonin uptake or indeed diminished using 2-Bromolysergic acid (BOL), a serotonin antagonist, applied to the hypothalamus. These results indicate that when activated, the ascending serotoninergic neurones increase blood pressure and this effect is mediated at least in part by the hypothalamus.

2.7.3. Others

Serotonin abnormalities have been tentatively linked to a number of other illnesses, particularly in the CNS. As previously mentioned in section 2.5.1. serotonin has been associated with depression. Treatment with amitriptyline, a drug which inhibits the re-uptake mechanism of 5HT from the synaptic cleft and therefore sustains the levels and actions of serotonin, causes a decrease in $5HT_2$ receptors, i.e down regulation, which correlates well with the therapeutic effect of the drug on the depression. These patients are thought to have supersensitive $5HT_2$ receptors (Peroutka *et al* 1980a).

Serotonin is also thought to play a role in Alzheimers disease, as these patients have degenerative serotoninergic neurones, and in anorexia nervosa and other appetite disorders, as fenfluramine which enhances release of serotonin from neurones induces anorexia and is used in the treatment of obesity (Rosser and Iverson 1986). Schizophrenia may also be due to a central serotonin disorder, as the hallucinogenic properties of LSD, a potent $5HT_2$ agonist, are similar to those experienced by schizophrenics.

Outwith the CNS in addition to its role in the Carcinoid Syndrome and hypertension, serotonin may play a role in migraine. Studies in these patients show abnormalities in the

storage and release of serotonin from the platelets (Hanington 1978). Treatment with methysergide and pizotifen, non selective serotonin antagonists, have proved somewhat successful.

Serotonin may be involved in many other conditions which are too numerous to describe here, however the reader is referred to the following references : Raynaud's phenomenon (Seibold 1985), pre-eclampsia (Weiner 1985) and acute respiratory failure (Hechtman 1985).

2.8. Objectives of project

It is clear that serotonin is an important compound found within the circulatory system and therefore has the potential to act on almost any part of the body. Although its action as a neurotransmitter and its role in the blood clotting process are well documented, the role of serotonin in the control of adrenal function and mineralocorticoid secretion has so far received relatively little attention.

The main objectives of this study were to establish a role for serotonin in the control of aldosterone secretion. Firstly, by identifying which serotonin receptor(s) are present in the zona glomerulosa and establish whether they may interact with the classical secretagogues of aldosterone. Secondly, to study the mechanism by which serotonin stimulates aldosterone secretion, with particular reference to cyclic AMP and calcium as intracellular messengers. Thirdly, to study the physiological importance of serotonin in stimulating aldosterone secretion and establish its mechanism of action *in vivo* and finally, to study the importance of serotonin as a trophic factor for the zona glomerulosa and establish if any changes observed are a direct action on the adrenal gland or are secondary to activation of the renin -angiotensin system or the hypothalamo-pituitary adrenal axis.

Chapter Three

Materials and Methods.

3.1. Introduction

This section describes the general methodology, specialised techniques and laboratory procedures used routinely throughout these studies. A brief review of the methods used in each chapter is given at the beginning of that chapter.

3.2. *In vitro* studies

3.2.1. Isolation of zona glomerulosa cells

The adrenal glands from female Wistar rats (180-220 g), killed by cervical dislocation, were removed and trimmed of surrounding fat. The capsules and adhering zona glomerulosa were removed following the method of Haning *et al* 1970, and incubated for 1 hour at 37°C in medium 199, pH 7.35-7.45, equilibriated in an atmosphere of 95% O_2 / 5% CO_2 and containing 2 mg/ml/rat collagenase and 2% (weight for volume, w/v) bovine serum albumin (BSA). The cells were disaggregated gently at 20 minute intervals using a wide mouthed 5 ml pipette. After 1 hour the dispersed cells were filtered through 100 µ gauze and washed 3 times by centrifugation for 15 minutes at 200g with resuspension of the cell pellet in 10 ml of medium 199. After the final spin the cells were resuspended in medium 199 containing 0.5% BSA (w/v), and an aliquot was removed for cell counting using a haemocytometer. On average this method produced a zona glomerulosa population with less than 5% contamination by zona fasciculata.

3.2.2. Cell counting

100 µl of a 0.2% (w/v) solution of trypan blue was added to 100 µl of the cell suspension. This was mixed thoroughly and a small aliquot placed on the counting chamber of a haemocytometer and allowed to diffuse. The cells were then viewed microscopically at a 400 fold magnification and those in the outside 4 and centre squares which excluded trypan blue, and therefore considered viable, were counted. This number was then corrected as follows to give the total number of cells.

Total cells = number counted within the squares x2 (trypan blue dilution factor) x 20 000 (converts volume of chamber to total volume of cells i.e 10 ml).

3.2.3. Static cell incubations

0.4 ml aliquots of the cell suspension, adjusted to contain on average 7.5 x 10^4 cells, were added to polyethylene vials containing 0.6 ml of medium 199 (0.5% w/v BSA) in which all the agonists / antagonists and other test substances had been dissolved to the required final concentration. Control incubates contained cells and medium 199 only. Each incubation was carried out in triplicate. The vials were mixed thoroughly and incubated for 1 hour at 37°C in a shaking water bath. After 1 hour they were placed on ice to prevent further steroid production and centrifuged at 20 000g for 2 minutes at 4°C. The supernatant was decanted and 500 µl removed for immediate processing for the cyclic AMP radioimmunoassay (RIA) as described later. The remainder was used for aldosterone estimation, also by RIA.

3.2.4. Potassium studies

The potassium concentration of the medium 199 (normally 3.9 mM) was increased by the addition of KCl. A compensatory adjustment was made to the sodium concentration by decreasing the volume of NaCl added. For the static incubations, the cells were resuspended after the final spin in medium 199 with the required potassium concentration and as before, 0.4 ml of cells were added to 0.6 ml of the same medium 199 containing all the test substances.

3.3. Radioimmunoassays for *in vitro* studies

3.3.1. Aldosterone radioimmunoassay

Aldosterone was measured by a highly specific direct RIA (Campbell *et al* 1981). 25 µl aliquots from the cell incubation extracts or 25 µl containing a known concentration of aldosterone, for the standard curve, were incubated in duplicate with 200 µl of phosphate

The counter plotted the best fit line through the logarithmic plot of counts per minute against the concentration of standard. The % free in each charcoal pellet for each standard could be calculated from the total number of counts added to each tube. The % bound (B) could then be calculated by subtracting the % free from 100%. Each assay had negligible non-specific binding. All standard curves depicted have been drawn logarithmically as $B/B_{o(\text{zero standard})}$ % versus the concentration of standard.

buffer containing ^{125}-Iodohistamine-3-mono-oxime-aldosterone (^{125} I-aldosterone) (3000-4000 counts per minute, cpm) and antisera (402 L) to aldosterone diluted to give a final titre of 1:100 000. The antibody was kindly provided by Dr F A O Mendelsohn, Australia.

The tubes were allowed to equilibrate overnight at 4°C. Separation of the bound and free radiolabel was carried out at 4°C by the addition of 0.6 ml of dextran coated charcoal followed by centrifugation at 2500g for 20 minutes, also at 4°C. The supernatant was aspirated and the charcoal pellet, containing the free fraction, counted in an LKB multiwell gamma counter.

The lower limit of detection for the assay was 0.07 nM and the working range for aldosterone measurement was 0.07 to 20 nM. A typical standard curve is shown in figure 3.1. The intra and interassay co-efficient of variation was 7 and 15% respectively.

3.3.2. Cyclic AMP radioimmunoassay

Cyclic AMP was measured by a highly specific direct RIA developed in this department by Dr I F Gow (Harper and Brooker 1975). Prior to RIA, 500 µl of the cell incubation extract was acidified by adding 5 µl of 20% (v/v) concentrated acetic acid and acetylated with 15 µl of a solution containing 2 parts triethylamine: 1 part acetic anhydride. 50 µl aliquots of the resulting cell incubation extract or 50 µl containing a known concentration of cyclic AMP for the standard curve were incubated in duplicate with 200 µl of acetate buffer containing ^{125}-I tyrosine-methyl-ester succinyl cyclic AMP (^{125} I-cyclic AMP) (3000-4000 cpm) and antisera (FB6) to cyclic AMP to give a final titre of 1:40 000. The tubes were allowed to equilibrate overnight at 4°C. Separation of bound and free radiolabel was carried out using dextran coated charcoal as described in the previous section. The lower limit of detection for the assay was 0.06 nM, and the working range of the assay was 0.06 to 32nM. A typical standard curve is shown in figure 3.2. The intra and inter assay co-efficient of variation was 8 and 16% respectively.

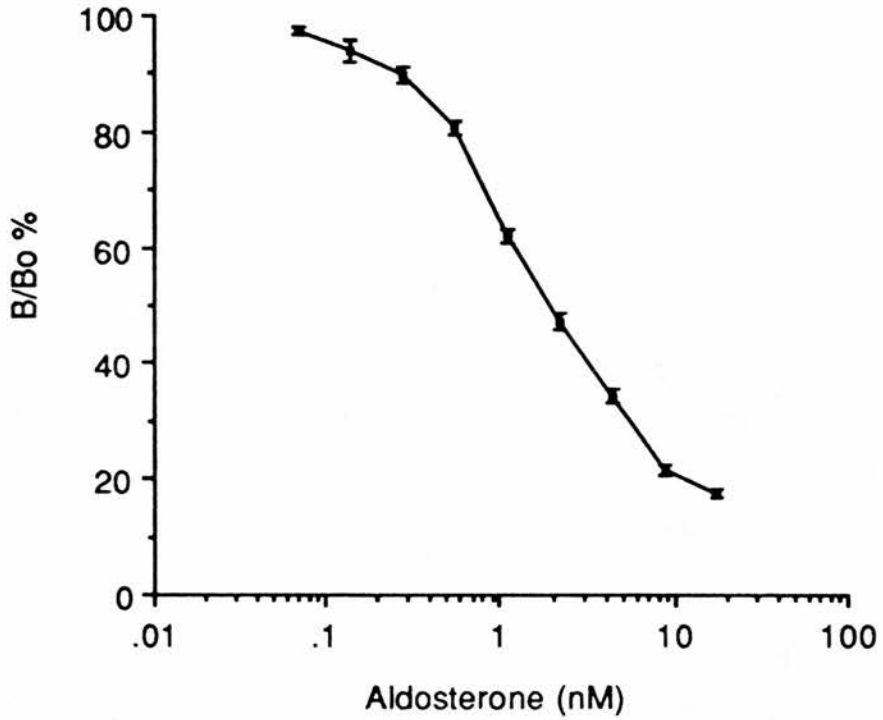


Figure 3.1. **A typical standard curve for the measurement of aldosterone (*in vitro*) by RIA.**

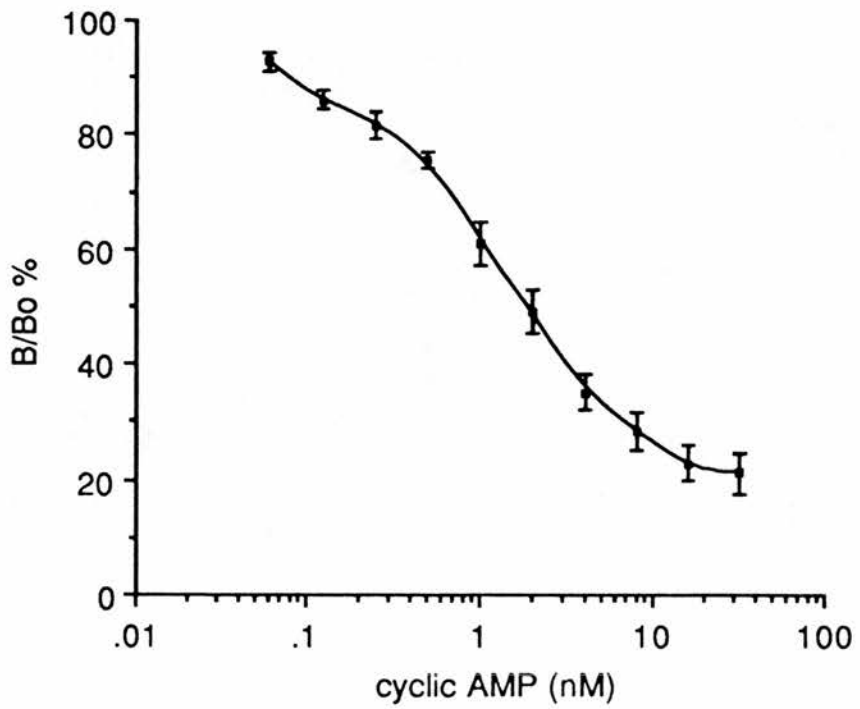


Figure 3.2. **A typical standard curve for the measurement of cyclic AMP (*in vitro*) by RIA.**

3.4. *In vivo* studies

3.4.1. Cannulation

Male Wistar rats (250-300 g) were anaesthetised by intra-peritoneal (i.p) injection of Hypnorm (0.05 ml / 100 g) and Hypnovel (0.05 ml / 100 g). The carotid artery was cannulated and the free end of the cannula threaded through to the back of the animal's neck using a trochar, where it was tied in position and kept closed by means of a small metal pin in the free end. The trochar was removed and the neck wound closed using metal clips. Immediately following surgery the cannulae were flushed with 20 µl of neat heparin (5000 units/ml) and then daily with 20 µl of neat heparin. The animals were allowed a 2 day post-operative recovery period before any experimental work commenced. The cannulae were used as a route of blood sampling throughout the experimental procedure.

3.4.2. Captopril pretreatment

Captopril was dissolved in the animals drinking water (0.5 mg/ml) and allowed *ad libitum* for 5 days prior to the cannulation and throughout the experimental procedure. Control animals received normal drinking water. Each animal consumed approximately 50 ml of water/day.

3.4.3. Dexamethasone pretreatment

1 ml of dexamethasone (15 µg/ml) dissolved in saline was administered i.p at 5 pm on the day prior to and at 9 am on the morning of the experimental procedure. Control animals received 1 ml of saline i.p.

3.4.4. Implantation of osmotic pumps

In the fifth study, 2 of the groups of animals were implanted with osmotic pumps, the same day as commencing captopril pretreatment, that is 5 days prior to the cannulation and 7 days prior to the experimental procedure.

The animals were anaesthetised as previously described. The skin of the back was shaved and a small incision was made. A subcutaneous pocket was made using a hemostat and the osmotic pump was inserted. The wound was closed by means of 2 metal clips. The pumps delivered either angiotensin II (3.5 µg /60 µl / hour) or 0.9% saline (60 µl / hour). The pumps were capable of delivering the required substance for up to 14 days.

3.4.5. Experimental procedure

All the experiments commenced at 9.30 am. A basal blood sample (time=0) was taken via the cannulae from all the animals immediately before i.p administration of 1ml of 5HTP (4 mg/ml) or saline (0.9% w/v), depending on the group . Further blood samples were taken at 45 minute intervals thereafter. A total of four blood samples including the basal were taken on the day of the experiment in addition to a 24 hour sample taken the following day. The cannulae were flushed immediately after drawing blood with 30 µl of heparin diluted 1:20 in saline. Only 250 µl of blood was drawn off at each time point to avoid volume depletion.

3.4.6. Measurement of blood pressure

Blood pressure was monitored using a Lectromed pressure transducer coupled to a carotid arteriole cannula. Mean arterial blood pressure (MABP) (mm Hg) was calculated using the formula ; $\frac{\text{systolic - diastolic}}{3}$ + diastolic.

3.4.7. Treatment of blood samples

225 µl of the blood was transferred to a tube containing 25 µl of ice cold ethylenediaminetetraacetic acid (EDTA) inhibitor. This was mixed thoroughly and centrifuged at 10 000g for 15 minutes. The plasma was removed and stored at -20^{o}C until measurement of PRA, corticosterone and aldosterone by RIA. Sodium and potassium were measured by Flame photometry at the Royal Hospital for Sick Children, Edinburgh.

20 µl of the remaining whole blood was added to 180 µl of a cocktail containing thrombin, chlorimipramine and pargyline. This was then left at 4°C for at least 2 hours to allow complete release of serotonin by the platelets and then centrifuged at 10 000g for 15 minutes. The supernatant was removed and stored at -20°C before estimation of 5HTP, 5HT and 5HIAA by HPLC.

3.5. Radioimmunoassays for *in vivo* studies

3.5.1. Plasma aldosterone radioimunnoassay

Plasma aldosterone was measured by a highly specific direct RIA (Campbell *et al* 1981). 25 µl of neat plasma or 25 µl of standard containing a known concentration of aldosterone for the standard curve were incubated in duplicate with 200 µl of phosphate citrate buffer containing ^{125}I-aldosterone (3000-4000 cpm) and antisera (402L) to aldosterone diluted to give a final titre of 1:100 000. The standards were made up in charcoal stripped rat plasma.

The tubes were allowed to equilibrate overnight at 4°C. Free and bound radiolabel were separated using dextran coated charcoal as previously described. The lower limit of detection of the assay was 0.195 nM and the working range was from 0.195 to 25 nM. A typical standard curve is shown in figure 3.3. The intra and inter assay co-efficient of variation was 7 and 15% respectively.

3.5.2. Plasma corticosterone radioimunnoassay

Plasma corticosterone was measured by a highly specific direct RIA (Al-Dujaili *et al* 1981). 10 µl of neat plasma was diluted with 90 µl of phosphate/citrate buffer. 25 µl of this dilution or 25 µl of standard containing a known concentration of corticosterone for the standard curve were incubated in duplicate with 400 µl phosphate/citrate buffer containing ^{125}I-iodohistamine-corticosterone-3-mono-oxime (^{125}I-corticosterone) (3000-4000 cpm) and antisera (R1B4) to corticosterone diluted to give a final titre of 1:40 000. Standards were made up in charcoal stripped plasma.

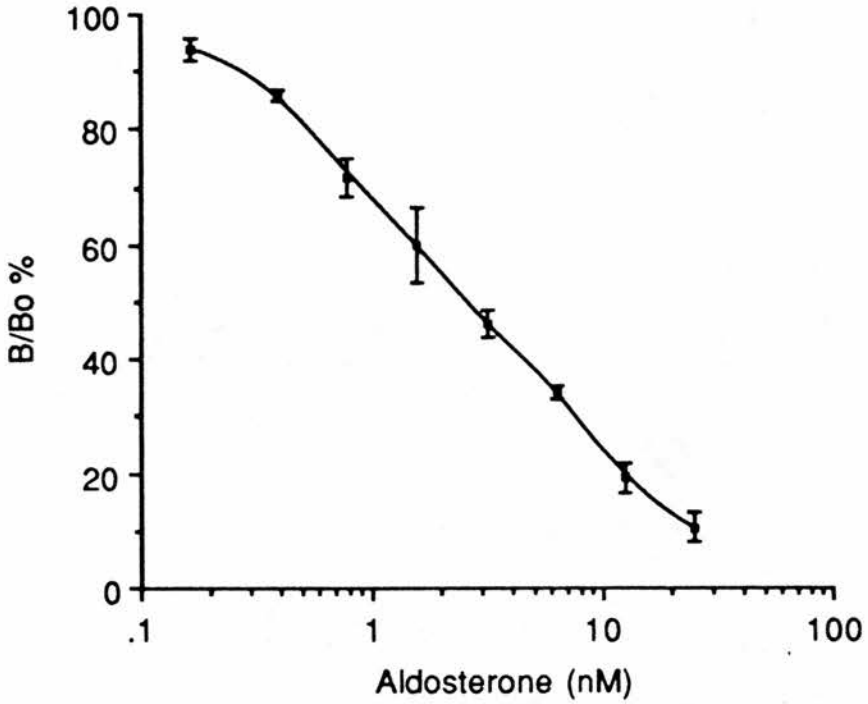


Figure 3.3. **A typical standard curve for the measurement of plasma aldosterone by RIA.**

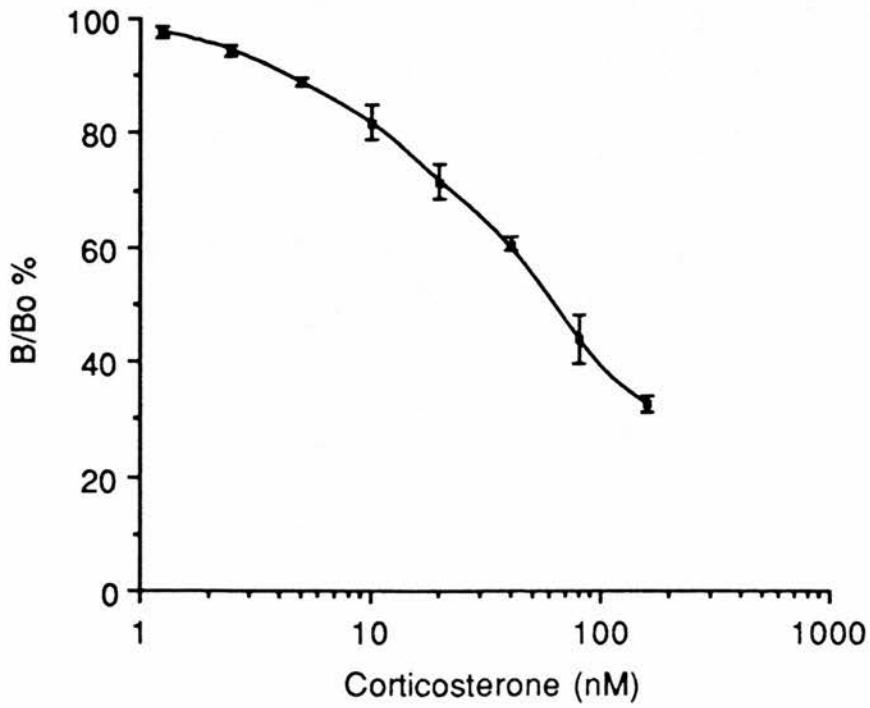


Figure 3.4. **A typical standard curve for the measurement of plasma corticosterone by RIA.**

The tubes were allowed to equilibrate overnight at 4°C. Free and bound radiolabel were separated using dextran coated charcoal as previously described. The lower limit of detection of the assay was 1.25 nM and the working range was from 1.25 to 160 nM. A typical standard curve is shown in figure 3.4. The results were corrected for the initial 10 fold dilution. The intra and inter assay co-efficient of variation was 8 and 14% respectively.

3.5.3. Plasma renin activity radioimunnoassay

Plasma renin activity (PRA) was evaluated by measuring angiotensin I generated from sheep substrate at 4 and 37°C. 10 µl of plasma was mixed with 190 µl of a premix containing 400 parts sheep substrate, 415 parts phosphate buffer, and 20 parts each of EDTA, British Anti-Lewisite (BAL) and 8-OH-quinoline. The mixture was then divided and one half incubated at 37°C for 4 hours, whilst the remaining half was maintained at 4°C. Angiotensin I was measured using a highly specific direct RIA. 25 µl of each of the plasma samples or 25 µl containing a known concentration of angiotensin I for the standard curve were incubated in duplicate with 200 µl of phosphate buffer containing ^{125}I-angiotensin I (3000-4000 cpm) and antisera to angiotensin I (R5B4) diluted to give a final titre of 1:10 000. Standards were made up in the premix solution.

The tubes were allowed to equilibrate overnight at 4°C. Free and bound radiolabel were separated using dextran coated charcoal as previously described. For this assay the results are expressed in grams rather than molar terms, this is in accordance with expression of PRA in many journals. The lower limit of detection of the assay was 0.5 ng/ml and the working range was from 0.5 to 64 ng/ml. A typical standard curve is shown in figure 3.5. The PRA was calculated by subtracting the concentration of angiotensin I in the sample maintained at 4°C from that of the sample incubated at 37°C. The results were corrected for the initial 20 fold dilution and the 4 hour incubation period. The intra and inter assay co-effcicient of variation was 8 and 16% respectively.

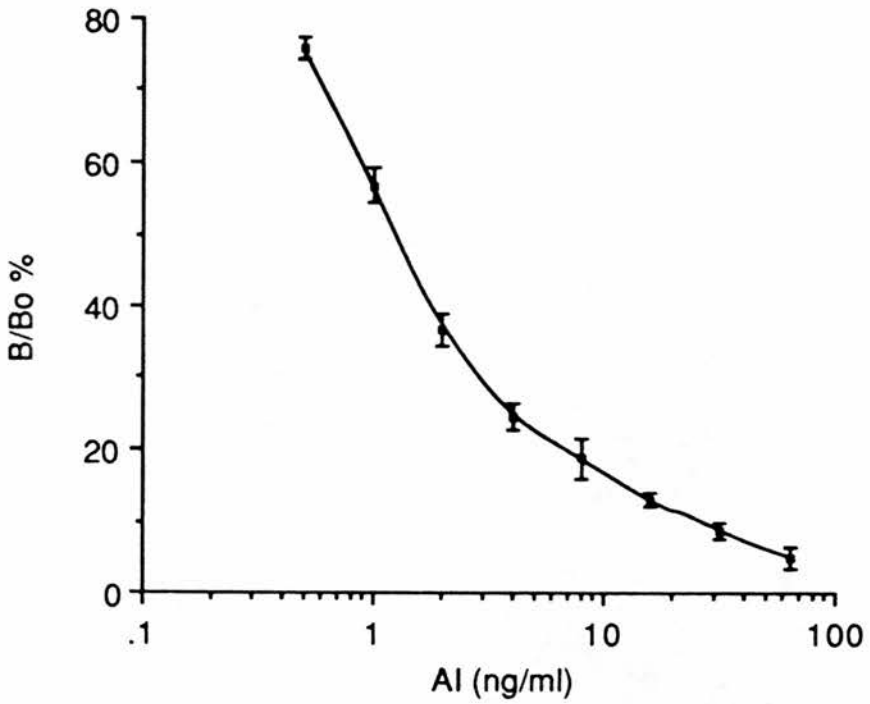


Figure 3.5. **A typical standard curve for the measurement of plasma angiotensin I by RIA for PRA.**

3.6. HPLC system for measurement of serotonin, 5HTP, AND 5HIAA

5HTP, 5HT and 5HIAA were measured in each serum sample using a high pressure liquid chromatography (HPLC) technique with a stationary phase C 18 column and electrochemical detection (Gow *et al* 1987).

120 µl of serum was mixed thoroughly with 15 µl of the internal standard N-methylserotonin (18.5 µM) and deproteinised with 15 µl of 15% (v/v) perchloric acid. After 15 minutes at 4°C the sample was centrifuged at 10 000g for 15 minutes at 4°C. The supernatant was removed and 20-100 µl injected into the HPLC system.

The mobile phase used in the system is given in section 3.11.12. The flow rate was 0.5 ml /minute and the electrochemical detection set at +0.7 volts with 5 nA full scale deflection.

25 µl of each standard, containing known concentrations of 5HT (700 nM), 5HTP (300 nM), 5HIAA (500 nM) and the internal standard N-methylserotonin (1.85 µM) were injected at regular intervals during the process in order to monitor the retention time and peak height, which is directly proportional to concentration, of each of the compounds throughout the day. The internal standard acted as a marker of % recovery. A typical HPLC elution profile for serotonin, its precursor and metabolite is shown in figure 3.6.

3.7. Calcium influx studies

3.7.1. Percoll density gradients

An 80% (v/v) stock solution of percoll was made by adding 80 ml of percoll to 20 ml of Krebs Ringer which was 5 times the normal concentration. 60, 40, 30 and 10% percoll solutions were then made from the stock solution by diluting it appropriately with Krebs Ringer of normal concentration. Discontinuous step gradients were made by layering 2 ml of 80 and 60, 24 ml of 40 and 2 ml of 30 and 10% percoll, sequentially into a large centrifuge tube using a peristaltic pump. Adrenal cells, isolated as described, and resuspended in a volume of 2 ml, were layered on top of the gradient. An identical gradient was loaded with 2 ml of Krebs

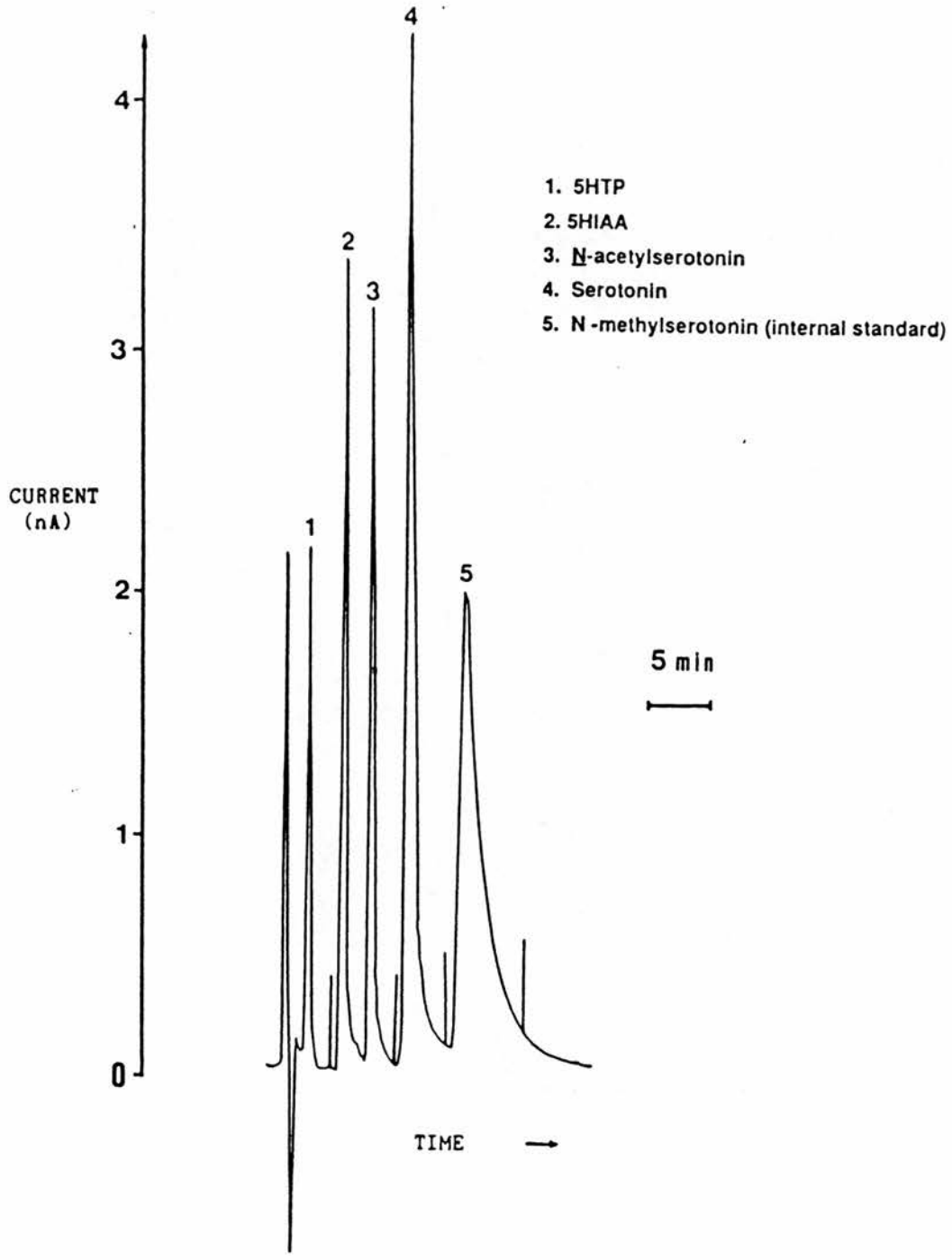


Figure 3.6. A typical HPLC elution profile of 5HTP, 5HT and 5HIAA.

Ringer containing red and blue marker beads of densities 1.062 and 1.076 g/ml respectively . The gradients were centrifuged at 800g for 20 minutes to allow separation of contaminating red blood cells, zona fasciculata cells and cellular debris from the glomerulosa cells. The glomerulosa cells were located between the blue and red marker beads, whereas the red blood cells were found at the bottom of the gradient. The cells were removed and washed twice by centrifugation for 10 minutes at 200g and resuspension in 10 ml Krebs Ringer to remove any percoll contamination.

3.7.2. Calcium influx experimental procedure

Following purification by percoll density gradient centrifugation, the cells were counted and adjusted to give 10^6 cells / 385 µl and incubated for 30 minutes at 37°C in Krebs buffer with 2% (w/v) BSA.

Following a modified version of the method of Mauger *et al* 1984. 385 µl of the cell suspension was added to 15 µl of $^{45}CaCl_2$ (5 µCi) and 100 µl of Krebs buffer (control) or 100 µl of stimulus dissolved in Krebs Ringer to the appropriate concentration. Where an antagonist was used, 50 µl of this was pre-incubated with the cells for 30 minutes at 37°C and the volume of buffer/stimulus was adjusted accordingly so that the final volume of each incubation was 500 µl. The specific activity of the $^{45}CaCl_2$ was 10 - 40 mCi / mg Ca^{2+}.

Following the addition of the cell suspension to the $^{45}CaCl_2 \pm$ agonist, a 100 µl aliqout of the incubation was removed at 15, 45, 75 and 105 second intervals. This was immediately diluted in 4 ml of ice cold Tris washing solution, filtered through a Whatman GF/C glass-fibre filter and washed a further 3 times with the washing solution. The radioactivity within the cells, i.e that associated with the filter, was counted in a β–counter using cocktail-T liquid scintillation fluid .

3.8. Morphology studies

3.8.1. Diets

Female Wistar rats of the same age and weight (200-220g) were maintained on one of the following diets for a period of 1-2 weeks. Each group of animals was allowed water *ad libitum* and consumed approximately 50 ml of water /day. The drinking water containing 5HTP, captopril, dexamethasone, 5HTP plus captopril or 5HTP plus dexamethasone was made up twice daily to avoid any degradation.

1. Normal	Normal chow diet. Normal drinking water.
2. 5HTP.	Normal chow diet. 5HTP dissolved in the drinking water (80 µg/ml).
3. Captopril	Normal chow diet. Captopril dissolved in the drinking water (0.5 mg/ml).
4. 5HTP + Captopril.	Normal chow diet. 5HTP (80 µg/ml) and captopril (0.5 mg/ml), dissolved in the drinking water.
5. Low sodium	Wholemeal diet containing 0.06% NaCl.
6. Normal sodium	Wholemeal diet containing 0.4% NaCl.
7. High sodium	Wholemeal diet containing 3% NaCl.
8. Dexamethasone	Normal chow diet. Dexamethasone dissolved in the drinking water (0.6 µg/ml).
9. Dexamethasone + 5HTP	Normal chow diet. Dexamethasone (0.6 µg/ml) and captopril (0.5 mg/ml) dissolved in the drinking water.

3.8.2. Adrenal sections

After the appropriate time period the animals were killed by cervical dislocation, the adrenal glands removed, trimmed of fat and snap frozen in isopentane cooled to -150°C in

liquid nitrogen. The glands were cut into 10 μ sections using a cryostat and placed on standard microscope slides. Approximately 2/3 sections were taken fron the centre of each gland.

3.8.3. Adrenal staining

To examine the zonation of the glands, the sections were stained by immersing the slides into the following solutions in order.

1. 60% (v/v) isopropyl alcohol
2. Oil red-o for 10 minutes
3. 60% (v/v) isopropyl alcohol
4. Distilled water
5. Haematoxylin for 2 minutes
6. Hot water (60°C)
7. Distilled water

The sections were then mounted in gelatin, and the width of the zona glomerulosa and zona fasciculata / reticularis was measured using a graticule attached to a light microscope. With 100 fold magnification 1 division of the graticule was equivalent to 10 μ. Five measurements were taken from around each section and averaged. Oil red-o is a lipid stain, whilst haematoxylin is specific for the nucleus.

3.8.4. Blood sampling

Blood samples from the animals maintained on the various diets were taken by cardiac puncture preceded by gentle cervical dislocation. This method ensured a relatively large volume of blood without using an anaesthetic which can significantly affect hormone secretion rates, and also avoided decapitation, the standard method of collecting large volumes of blood.

3.9. Radioimmunoassays for morphology study

3.9.1. Plasma angiotensin II radioimmunoassay

Plasma angiotensin II was measured by a highly specific RIA (Morton and Webb 1985). Blood was collected into ice cold EDTA / phenanthrolene inhibitor (20 parts blood: 1 part inhibitor) and mixed thoroughly. The sample was centrifuged at 2500g for 30 minutes at 4°C. The plasma was removed and a measured volume loaded on to a Sep-pak C_{18} cartridge already primed with 5 ml of methanol followed by 5 ml of water. After the plasma sample the cartridge was washed with a further 5 ml of water. The angiotensin II attached to the column was eluted with 2.2 ml of methanol (80% v/v). The eluate was collected and dried down at 37°C under a stream of air. The resulting extract was resuspended in 0.5 ml of tris buffer.

The angiotensin II in each extract was measured by a highly specific RIA. 50 µl of the extract or 50 µl containing a known concentration of angiotensin II for the standard curve were incubated in duplicate with 200 µl of tris buffer containing ^{125}I-angiotensin II (3000-4000 cpm) and antisera to angiotensin II (R2B5) diluted to give a final titre of 1:300 000. Standards were made up in tris buffer .

The tubes were allowed to equilibrate overnight at 4°C. Free and bound radiolabel were separated using dextran coated charcoal as previously described. The lower limit of detection for the assay was 0.02 nM and the working range was 0.02 to 10.24 nM. A typical standard curve is shown in figure 3.7. The intra and inter assay co-efficient of variation was 7 and 17% respectively.

In parallel, the angiotensin I concentration in each tube was also measured using a similar protocol to the angiotensin I measurement for the PRA assay, with the exception that the standards were made up in tris buffer. As the angiotensin II antisera cross reacts with angiotensin I by 0.5%, this was corrected for, as was a 95% recovery rate from the Sep-pak cartridge and the volume of plasma initially utilsed.

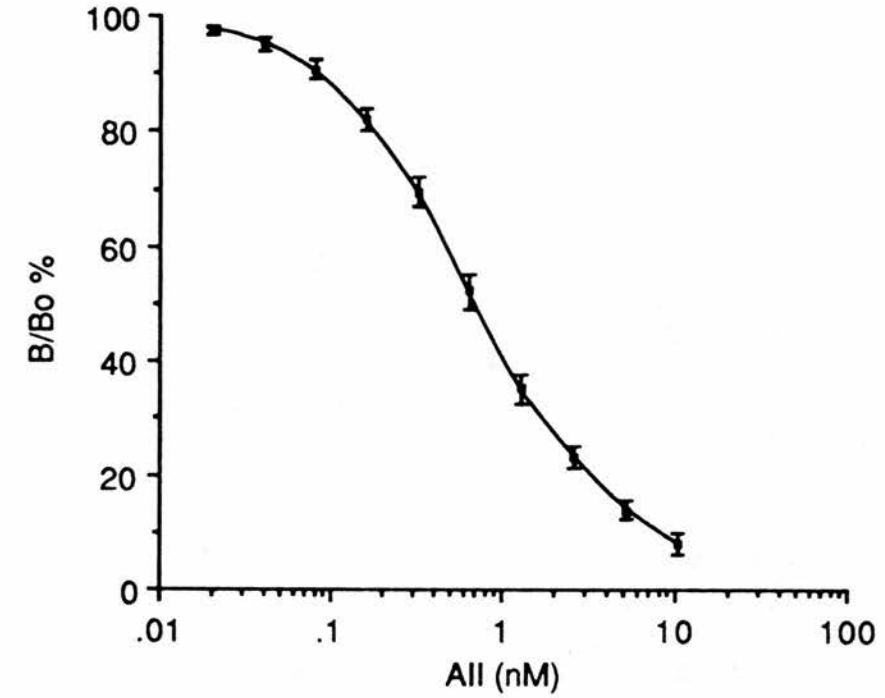


Figure 3.7. **A typical standard curve for the measurement of plasma angiotensin II by RIA.**

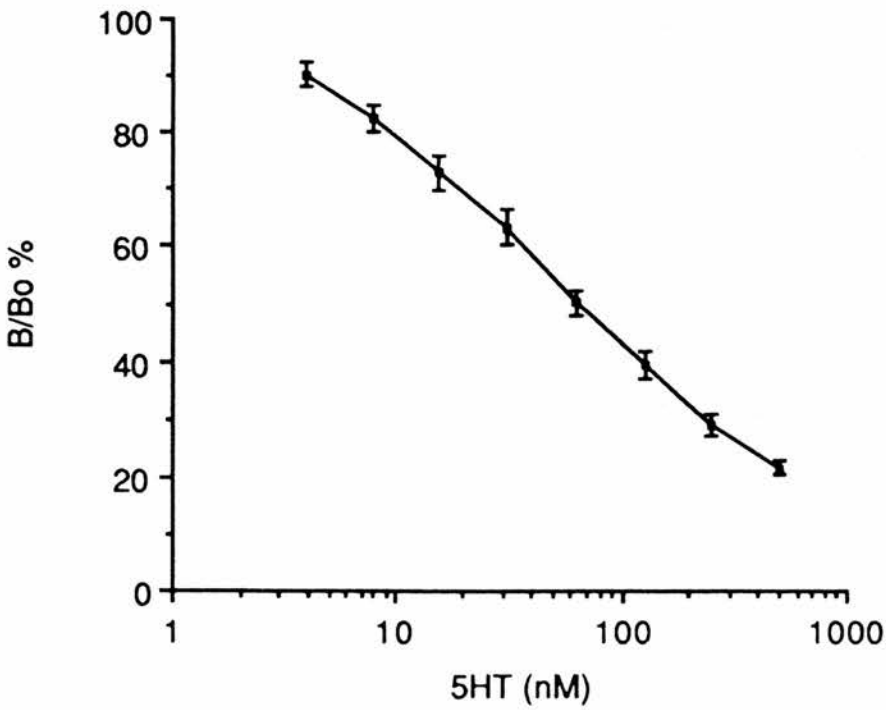


Figure 3.8. **A typical standard curve for the measurement of serum serotonin by RIA.**

3.9.2. Serum serotonin radioimmunoassay

Serum serotonin was measured using a highly specific RIA (Gow *et al* 1987). Blood was taken from the animal and allowed to clot at 4°C for 4 hours. It was then centrifuged at 2500g for 15 minutes at 4°C. 10 µl of the serum was added to 900 µl of citrate buffer and 900 µl of the resulting solution was deproteinised by adding 100 µl of perchloric acid (15% v/v) containing 2 mM cysteine, followed by centrifugation for 30 minutes at 1720g and 4°C. 600 µl of the supernatant was removed and acetylated with N-acetylsuccinimide (1 mg). The solution was then neutralised with 150 µl of sodium hydroxide (0.5 M) and the excess acetylating agent mopped up by adding 150 µl of glycine (16.6 µM). 200 µl of this or 200 µl of standard containing a known concentration of serotonin for the standard curve was then incubated in duplicate with 300µl of a pre-mix solution containing ^{125}I-glycyl-tyrosine N-succinamyl serotonin (^{125}I-5HT) (3000-4000 cpm), antisera to serotonin (R16B4), non immune rabbit serum (NIRS) and donkey anti-rabbit serum (DARS), diluted in citrate buffer, the final concentration of which were 1:25, 1:20 000, 1:4000 and 1:400 respectively.

The tubes were allowed to equilibrate overnight at 4°C. Separation of the bound and free fractions was by centrifugtion for 25 minutes at 1720g and 4°C. The supernatant was removed and the pellet containing the bound fraction counted in a gamma counter. The lower limit of detection of the assay was 3.9 nM and the working range was 3.9 to 500 nM. A typical standard curve is shown in figure 3.8. The intra and inter assay co-efficient of variation was 8 and 17 % respectively. The results were corrected for the number of dilutions carried out.

3.9.3. Plasma aldosterone radioimmunoassay

The radioimmunoassay for measurement of plasma aldosterone concentration has already been described in section 3.5.1.

3.9.4. Plasma renin activity radioimmunoassay

The radioimmunoassay for PRA has already been described in section 3.5.3.

3.10. Source of materials

3.10.1. Cell isolation

Medium 199	Flow Laboratories, Irvine, Scotland.
BSA	Miles Laboratories, Slough, England.
Collagenase	Worthington Biochemical Corp, USA.
Trypan blue stain	Sigma Chemical Company Ltd, Poole, England.
100 μ gauze	Henry Simon Ltd, Stockport, England.
Percoll	Sigma Chemical Company Ltd, Poole, England.
Percoll density marker beads	Pharmacia Ltd, Milton Keynes, England.
Salts for buffer solutions	BDH Ltd, Poole, England.
Glucose	BDH Ltd, Poole, England.

3.10.2. Stimuli

5HTP	Sigma Chemical Company Ltd, Poole, England.
5HT	Sigma Chemical Company Ltd, Poole, England.
Angiotensin II	Universal Biologicals, Cambridge, England.
db cyclic AMP	Sigma Chemical Company Ltd, Poole, England.
A23187 (calcium ionophore)	Sigma Chemical Company Ltd, Poole, England.
ACTH (Synacthen)	Ciba Laboratories, Horsham, England.

3.10.3. Antagonists/Drugs/inhibitors

TMB-8	Sigma Chemical Company Ltd, Poole, England.
Trifluoperazine	Sigma Chemical Company Ltd, Poole, England.
Saralasin	Universal Biologicals, London, England.

Nifedipine	Sigma Chemical Company Ltd, Poole, England.
Verapamil	Abbott Laboratories Ltd, Kent, England.
Ketanserin	Janssen Pharmaceuticals, Wantage, England.
Methysergide	Sandoz Pharmaceuticals, Middlesex, England.
Mesulergine	Sandoz Pharmaceuticals, Middlesex, England.
Cyanopindolol	Sandoz Pharmaceuticals, Middlesex, England.
ICS 205/930	Sandoz Pharmaceuticals, Middlesex, England.
Captopril	E.R Squibb, Moreton, England.
Dexamethasone	Organon Laboratories Ltd, Cambridge, England.
Pertussis toxin	Sigma Chemical Company Ltd, Poole, England.

3.10.4. Morphology

Haematoxylin	Sigma Chemical Company Ltd, Poole, England.
Oil red-o	Sigma Chemical Company Ltd, Poole, England.
Cryo-M-bed	Taab Laboratories, Berkshire, England.
Cryo-Jet	BDH Ltd, Poole, England.
Iso-pentane	BDH Ltd, Poole, England.
Mercuric oxide	BDH Ltd, Poole, England.
Sodium iodate	BDH Ltd, Poole, England.
Glycerol gelatin	Sigma Chemical Company Ltd, Poole, England.
Liquid nitrogen	British Oxygen Company, Surrey, England.

3.10.5. HPLC

5HIAA	Sigma Chemical Company Ltd, Poole, England.
N-methyl-5HT	Aldridge Chemical Co. Ltd, Gillingham, England.
Perchloric acid	May and Baker, Manchester, England.
HPLC grade water	Rathburn Chemicals Ltd, Walkerburn, Scotland.

HPLC grade methanol	Rathburn Chemicals Ltd, Walkerburn, Scotland.
Salts for mobile phase	BDH Ltd, poole, England.

3.10.6. Radiolabelled calcium studies

GF/C Filters	Whatman International Ltd, Maidstone, England.
$^{45}CaCl_2$	Amersham International plc, England.
Cocktail T scintillation fluid	BDH Ltd, poole, England.

3.10.7. Blood Collection

Thrombin	Sigma Chemical Company Ltd, Poole, England.
Pargyline	Sigma Chemical Company Ltd, Poole, England.
Phenanthrolene	Sigma Chemical Company Ltd, Poole, England.
EDTA	BDH Ltd, Poole, England.
Sep-pak C_{18} cartridges	Millipore/Waters Ltd, Edinburgh, Scotland.
Clorimipramine	Ciba Laboratories, Horsham, England.

3.10.8. Anaesthetics

Hypnorm	Janssen Pharmaceuticals Ltd, Oxford, England.
Hypnovel	Roche Pharmaceuticals Ltd, Herts, England.

3.10.9. Miscellaneous

Heparin	Leo Laboratories, Bucks, England.
DMSO	Sigma Chemical Company Ltd, Poole, England.
EGTA	BDH Ltd, Poole, England.
Ethanol	BDH Ltd, Poole, England.
Gelatin	Sigma Chemical Company Ltd, Poole, England.

Dextran T-70	Pharmacia Ltd, Milton Keynes, England.
BAL	Sigma Chemical Company Ltd, Poole, England.
8-OH-quinoline	Sigma Chemical Company Ltd, Poole, England.
Aracis oil	Hospital Pharmacy.
Benzyl benzoate	Sigma Chemical Company Ltd, Poole, England.
Perchloric acid	BDH Ltd, Poole, England.
DARS	Scottish antibody production unit, Carluke, Scotland.
NIRS	Scottish antibody production unit, Carluke, Scotland.
Cysteine	Sigma Chemical Company Ltd, Poole, England.
Glycine	Sigma Chemical Company Ltd, Poole, England.
Osmotic pumps	Alzet, Alza corp, Bucks, England.
N-acetylsuccinimide	Synthesised by Dr IF Gow in this laboratory. See Gow *et al* 1987.

3.11. Buffers / Inhibitors / Solutions etc

The constituents of all the solutions used throughout these studies are given below. Standard abbreviations or chemical names are used.

3.11.1. Medium 199

1 sachet of medium 199 was dissolved in 100 ml of distilled water and a 15 ml aliquot was added to 210 ml of salt solution containing $NaHCO_3$, $MgSO_4$, KH_2PO4, $CaCl_2$ and NaCl. The final concentration of ions in the medium in mM were; sodium 145, potassium 3.9, calcium 2.5, magnesium 1.2, bicarbonate 25, chloride 128, phosphate 1.2, and sulphate 1.2. The other constituents of medium 199 are too numerous to describe, but are given in page 55 of the Flow Laboratories catalogue.

3.11.2. Medium 199 with altered potassium concentration

When potassium concentration was increased, by the addition of KCl, a compensatory adjustment was made to the NaCl added, which was decreased by the same concentration.

3.11.3. Phosphate buffer

This solution contained 80 mM Na_2HPO_4, 20 mM KH_2PO_4, and 15 mM NaN_3, made up in distilled water. The pH was adjusted to 7.4

3.11.4. Dextran coated charcoal

6.25 g of charcoal, 0.4 g of gelatin predissolved in 20 ml of warm phosphate buffer and 0.62 g of dextran were added to 1 litre of phosphate buffer. The pH was adjusted to 7.4.

3.11.5. Sodium acetate buffer

This solution was made from 120 ml of acetic acid (0.1 M) and 80 ml of sodium acetate (0.1 M). The pH was adjusted to 4.75.

3.11.6. EDTA inhibitor for blood collection

This solution contained 27 mM EDTA, made up in 0.9% (w/v) saline.

3.11.7. Cocktail for blood collection

This solution contained 1.1 μM chlorimipramine, 11.1 μM pargyline and 1.1 units of thrombin /ml, made up in distilled water.

3.11.8. Phosphate / citrate Buffer

This solution contained 20 mM citric acid and 12 mM NaH_2PO_4. The pH was adjusted to 4.

3.11.9. EDTA for angiotensin I RIA

This solution contained 270 mM EDTA, made up in distilled water.

3.11.10. BAL

This solution contained15 ml Aracis oil, 242 µl 2-3 dimercapto-propan-1-ol and 535 µl benzyl benzoate.

3.11.11. 8-OH-Quinoline

This solution contained 454 mM 8-hydroxyquinoline, made up in distilled water.

3.11.12. HPLC mobile phase

This solution was made up in 80% water and 20% methanol and contained 130 mM NaH_2PO_4, 1 mM EDTA, and 2.4 mM octane sulphonic acid which was previously dissolved in 8ml of acetic acid (0.5 M).

3.11.13. Krebs Ringer (5 times normal concentration)

This solution contained 600 mM NaCl, 13 mM KCl, 6.2 mM $CaCl_2$, 5.9 mM KH_2PO_4, 5.9 mM $MgSO_4$ and 124 mM $NaHCO_3$, made up in distilled water. The pH was adjusted to 7.4.

3.11.14. Krebs Ringer (normal concentration)

This solution contained 120 mM NaCl, 2.6 mM KCl, 1.2 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$ and 25.8 mM $NaHCO_3$, made up in distilled water. The pH was adjusted to 7.4.

3.11.15. Tris washing solution

This solution contained 144 mM NaCl, 5mM $CaCl_2.2H_2O$, 5 mM Tris and 5 mM HCl. The pH was adjusted to 7.4.

3.11.16. EDTA / phenanthroline inhibitor

This solution contained 125 mM EDTA and 23 mM phenanthroline, previously dissolved in 1 ml ethanol, made up in distilled water.

3.11.17. Tris buffer

This solution contained 30 mM Tris and 15 mM NaN_3 made up in 30mM HCl. The pH was adjusted to 7.4.

3.11.18. Citrate buffer

This solution contained 100 mM citric acid, 300 mM NaOH, 1 mM EDTA and 1 g/ litre of gelatin, made up in distilled water. The pH was adjusted to 6.2.

3.11.19. Haematoxylin

1 g of hamatoxylin was dissolved in 10 ml of ethanol. 20 g of Al.K $(SO_4)_2.12\ H_2O$ was dissolved in 200 ml of hot distilled water. The two solutions were mixed and 0.5 g of mercuric oxide and 0.25 g of sodium iodate added.

3.11.20. Oil red-o

1 g of oil red-o was added to 200 ml of isopropyl alcohol. The solution was diluted by 40% with distilled water immediately before use.

3.11.21. Charcoal stripped plasma

1 g of charcoal plus 10 ml of plasma was mixed overnight and then spun at 2500g for 1 hour. The plasma was then filtered through Whatman number 1 paper.

Chapter Four

Identification and Characterisation of Adrenal Serotonin Receptors.

4.1. Introduction

Three main groups of serotonin receptors have been identified and characterised within the CNS and cardiovascular system, and these have been named the $5HT_1$, $5HT_2$ and $5HT_3$ binding sites. More recently, the availability of a number of new antagonists has clearly demonstrated the heterogeneity of the $5HT_1$ site, resulting in its sub-classification into the $5HT_{1a}$, $5HT_{1b}$, $5HT_{1c}$ and $5HT_{1d}$ receptor sites. However, very little attention has focussed on the serotonin receptors within the adrenal zona glomerulosa which responds to serotoninergic stimulation by increasing aldosterone secretion.

In order to define more clearly the serotonin receptor type(s) which mediate the steroidogenic response we have employed a wide range of serotonin antagonists and studied their effect on aldosterone secretion by isolated rat adrenal zona glomerulosa cells. In addition, their specificity was screened by observing their effects on the major aldosterone secretagogues i.e angiotensin II, ACTH and potassium.

4.2. Aims of study

1. To define which serotonin receptor(s) mediate the aldosterone response to serotonin, by studying the effects of a wide range of serotonin antagonists, which have a high affinity for specific serotonin receptors, on the aldosterone dose response to serotonin in isolated zona glomerulosa cells.
2. To assess the specificity of the antagonists by examining their effects on the aldosterone response to angiotensin II, ACTH and potassium .
3. To examine a possible interaction between the adrenal serotonin receptor and the angiotensin II receptor.

4.3. Materials and methods

The methods used in this chapter have been previously described in chapter 3

sections 3.2 to 3.3.2.

Briefly, rat adrenal zona glomerulosa cells were isolated and incubated with either

1. No stimulus (basal control).
2. 5HT (10^{-8}, 10^{-6} M).
3. Angiotensin II (10^{-8}, 10^{-10} M).
4. ACTH (10^{-9}, 10^{-11} M) or
5. Potassium (5.9, 8.4, 13.2 mM) in the presence or absence of one of the 5 serotonin antagonists at a concentration of 10^{-6} M. Following a 1 hour incubation period and removal of the cells, aldosterone and cyclic AMP were measured by RIA.

4.4. Serotonin antagonists

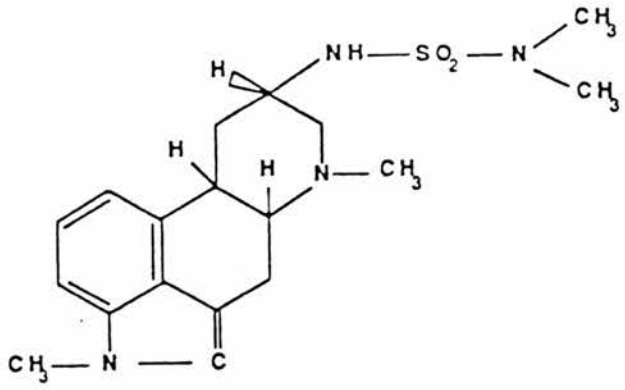
5 serotonin antagonists, covering the entire range of serotonin receptors were utilised in this study. The chemical structures, molecular weights, solubility and receptor specificity of ketanserin, methysergide, mesulergine, cyanopindolol and ICS 205/930 are illustrated in figure 4.1 and table 4.1.

4.5. Graphical Illustration

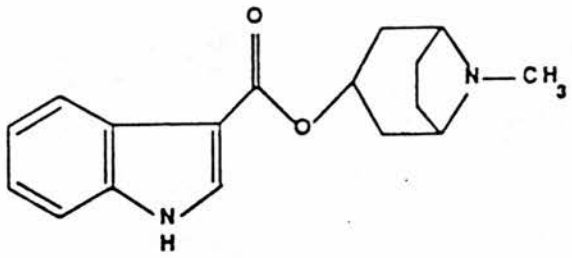
The graphs illustrated are single experiments incubated in triplicate and are representative of the general trends observed in each type of experiment. Each experiment shown was repeated at least once and on most occassions twice.

Although the cell number remained constant, small variations in basal cyclic AMP and aldosterone secretion were observed between experiments. For this reason aldosterone and cyclic AMP secretion are expressed as stimulation ratio $\pm$ standard error mean (SEM) in relation to the basal secretion of the control samples which have been given a value of 1. Variations in the responsiveness of the cells to a particular stimulus were also observed between experiments. These variations in basal secretion and responsiveness have been reported by other workers and are the result of differing degrees of cellular damage caused by

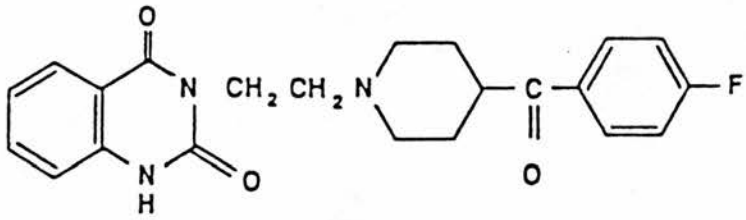
Mesulergine



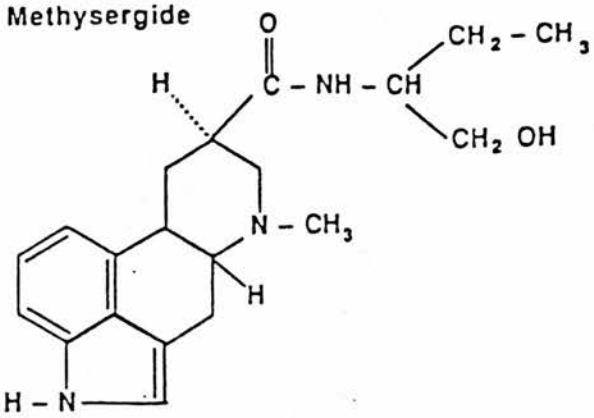
ICS 205/930



Ketanserin



Methysergide



Cyanopindolol

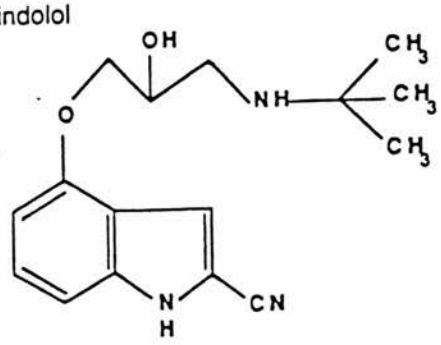


Figure 4.1. The chemical structure of the serotonin. antagonists.

Table 4.1

The properties of the serotonin antagonists

Antagonist	Mol. Wt.	Solubility	Receptor Specificity
Mesulergine	362.5	Saline	$5HT_{1c}$
Methysergide	460	Saline	$5HT_{1/2}$
Ketanserin	545.5	Saline	$5HT_2$
ICS 205/930	320.8	Saline	$5HT_3$
Cyanopindolol	287.3	Et OH	$5HT_{1a/1b}$

the harsh collagenase digestion (Vinson *et al* 1985).

The mean basal aldosterone secretion in 10 individual experiments was 1.212 ± 0.251 nM. The mean basal cyclic AMP secretion in 8 individual experiments was 0.431± 0.125 nM.

4.6. Statistical analysis

Statistical significance was calculated in the individual experiments by Students' t-test for unpaired samples. A p value of <0.05 was considered significant. NS indicates non significance. The p values in this section are illustrated in the figures and are not quoted in the text. *, ** and *** on the figures indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

4.7. Results

4.7.1. The cyclic AMP and aldosterone response to serotonin *in vitro*

This preliminary study illustrates the changes in cyclic AMP and aldosterone secretion in isolated rat adrenal zona glomerulosa cells following stimulation by serotonin.

Figure 4.2 shows a typical aldosterone dose response to serotonin (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) in the rat zona glomerulosa cell preparation used routinely throughout these studies. 10^{-8} M serotonin was sufficient to cause a significant increase in aldosterone secretion. Maximal stimulation was acheived by 10^{-6} M serotonin which caused a 3 fold increase in steroid output. In subsequent experiments only 2 concentrations of serotonin were studied; 10^{-8} and 10^{-6} M.

Figure 4.3 shows the corresponding cyclic AMP response to serotonin. Cyclic AMP output was not significantly increased at 10^{-8} M serotonin, although aldosterone secretion was elevated. 10^{-7} M serotonin caused a significant increase in cyclic AMP secretion and 10^{-6} M serotonin, which caused maximal steroidogenesis, caused a 3 fold increase in cyclic AMP.

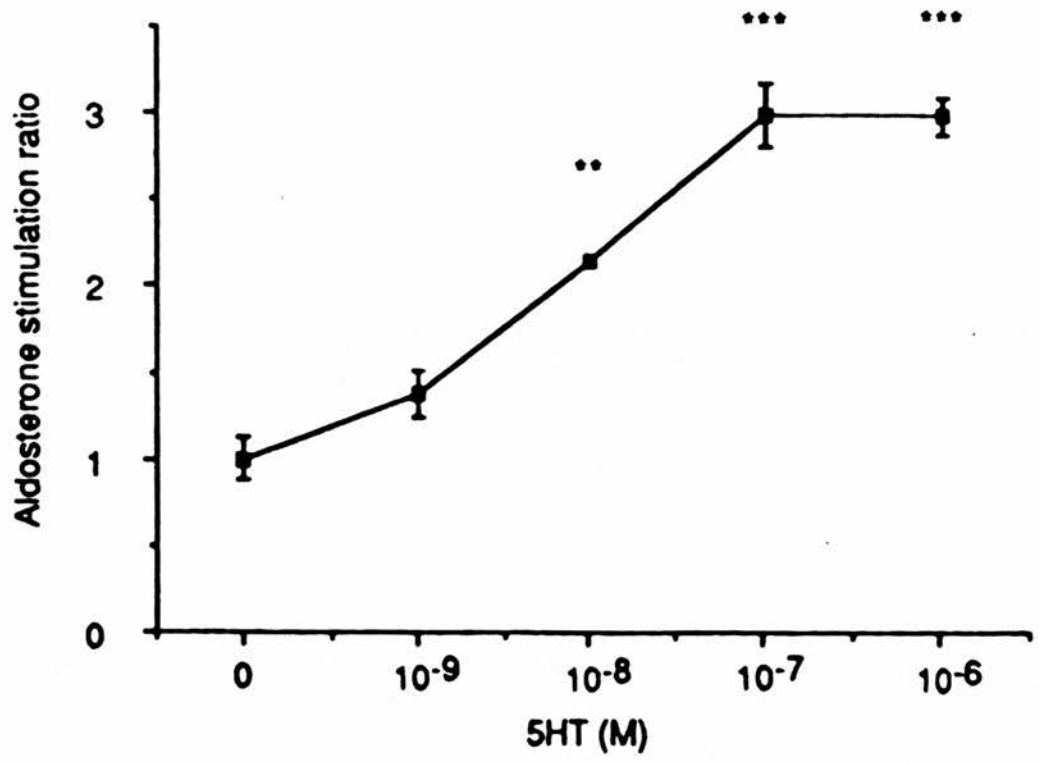


Figure 4.2. **A typical aldosterone response to serotonin.** (n=3)

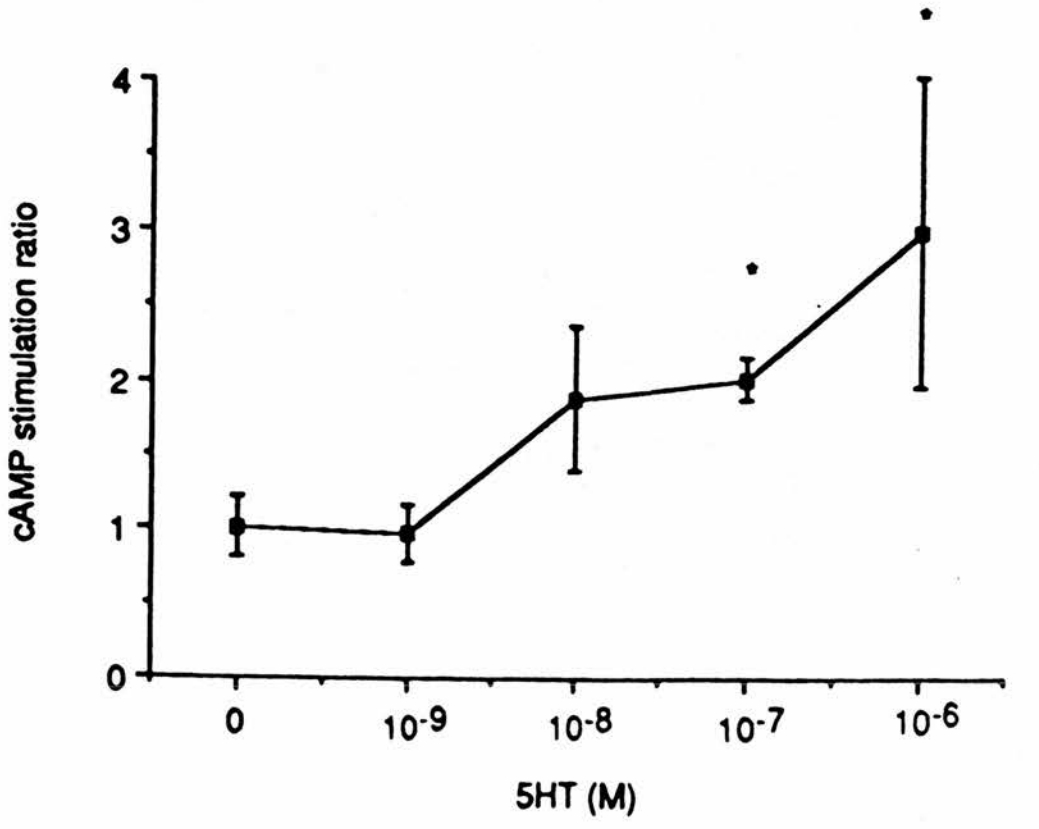


Figure 4.3. **A typical cyclic AMP response to serotonin.** (n=3)

4.7.2. The effects of the serotonin antagonists on the aldosterone and cyclic AMP dose response to serotonin *in vitro*

Having clarified that rat zona glomerulosa cells do respond to serotonin, an attempt was made to identify and characterise the adrenal zona glomerulosa receptor(s) for serotonin, by examining the effect of a wide range of antagonists specific for different serotonin receptors, on the aldosterone and cyclic AMP response to serotonin.

Figure 4.4 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of either 10^{-6} M ketanserin, methysergide, mesulergine, cyanopindolol or ICS 205-930. The receptor specificity and properties of these drugs have already been described in figure 4.1 and table 4.1.

Basal aldosterone secretion was significantly inhibited in the presence of methysergide, ketanserin and mesulergine. Cyanopindolol and ICS 205-930 showed no significant effect. At 10^{-8} M serotonin, all the antagonists caused significant inhibition of aldosterone secretion. With the exception of cyanopindolol and ketanserin, which failed to show any effect, all the antagonists sustained their inhibitory properties at 10^{-6} M serotonin.

The combined % inhibition of each of the antagonists on the aldosterone response to serotonin from the means of three separate experiments is shown in table 4.2. This clearly indicates that purely on the basis of % inhibition, mesulergine, methysergide and ketanserin are by far the most effective antagonists.

Figure 4.5 shows the corresponding cyclic AMP dose response to serotonin in the presence and absence of each of the serotonin antagonists (10^{-6} M). None of the antagonists significantly inhibited basal cyclic AMP secretion. At 10^{-8} M serotonin, all the antagonists significantly inhibited cyclic AMP secretion, an effect which was sustained at 10^{-6} M serotonin.

The combined % inhibition of each of the antagonists from the means of 2 separate

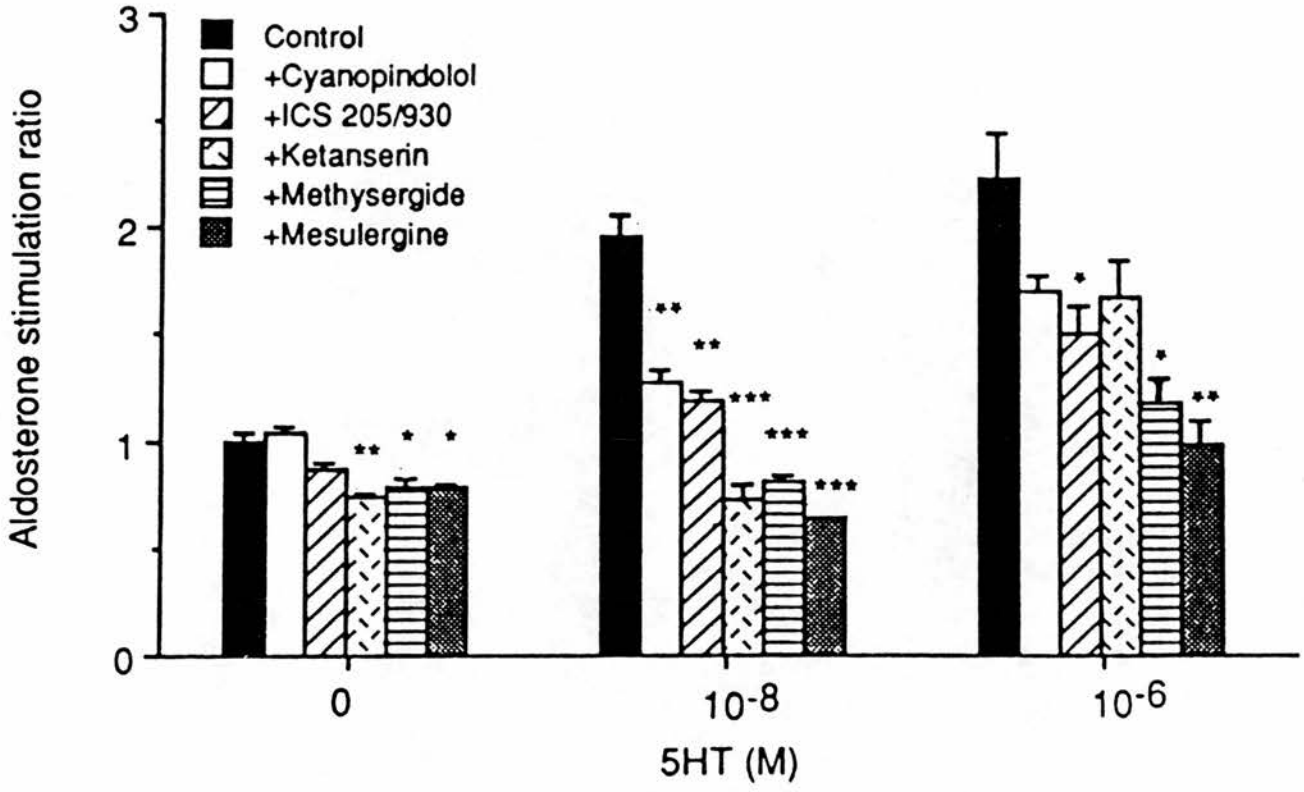


Figure 4.4.

The effect of serotonin antagonists (10^{-6}M) on the aldosterone dose response to serotonin.

* p<0.05 versus control
** p<0.01 versus control
*** p<0.001 versus control

Table 4.2.

The combined % inhibition ± SEM of each antagonist on the aldosterone response to serotonin from 3 experiments

	5HT Concentration (M)		
Antagonist	**0**	**10^{-8}**	**10^{-6}**
Mesulergine	39 ± 6	78 ± 11	50 ± 14
Methysergide	36 ± 4	70 ± 7	39 ± 8
Ketanserin	42 ± 6	57 ± 11	22 ± 4
ICS 205/930	8 ± 0.5	27 ± 6	14 ± 4
Cyanopindolol	---------	20 ± 4	8 ± 2

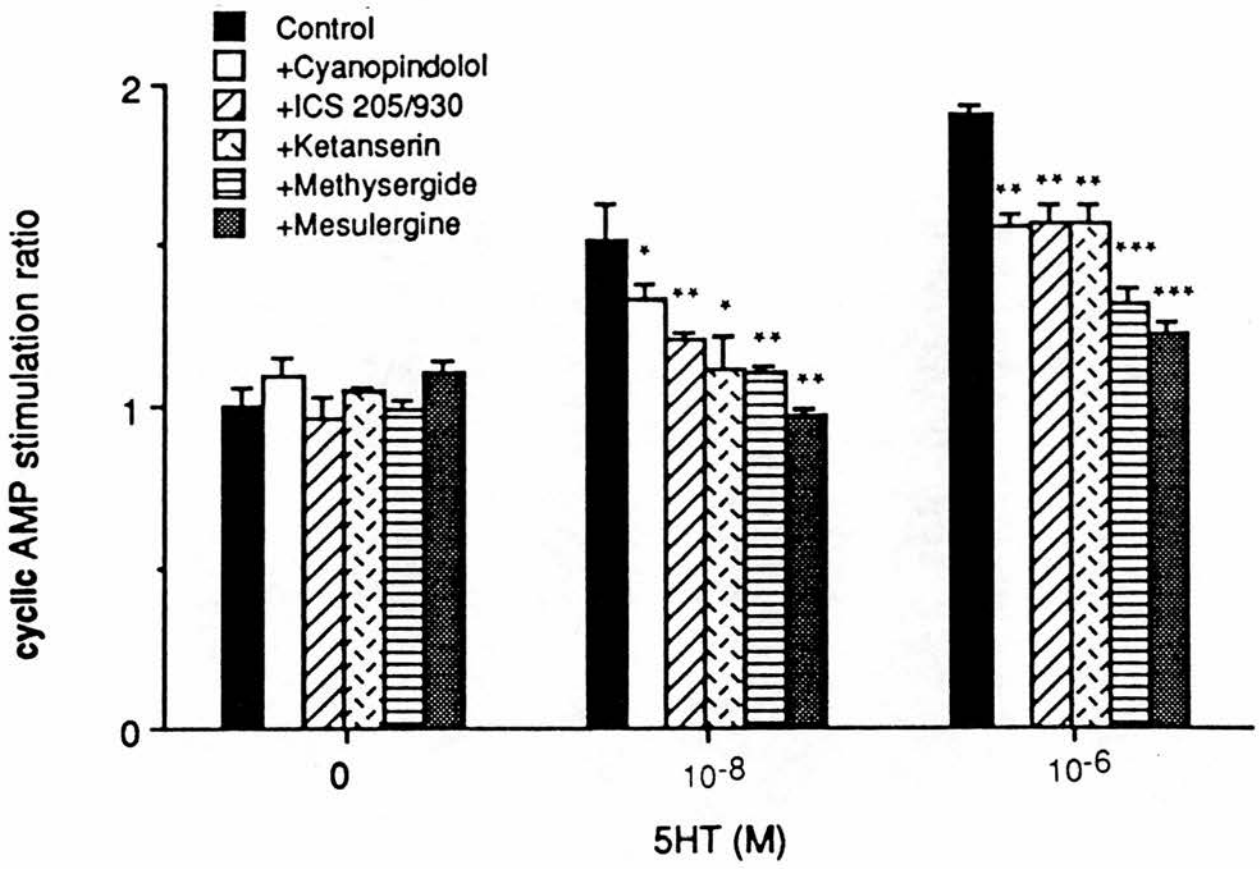


Figure 4.5.

The effect of serotonin antagonists (10^{-6}M) on the cyclic AMP dose response to serotonin.

* $p<0.05$ versus control
** $p<0.01$ versus control
*** $p<0.001$ versus control

Table 4.3.

The combined % inhibition ± SD of each antagonist on the cyclic AMP response to serotonin from 2 experiments

	5HT Concentration (M)		
Antagonist	**0**	**10^{-8}**	**10^{-6}**
Mesulergine	25 ± 12	41 ± 5	41 ± 8
Methysergide	9 ± 0.7	36 ± 6	36 ± 8
Ketanserin	13 ± 2	40 ± 10	26 ± 6
ICS 205/930	13 ± 1	37 ± 11	22 ± 4
Cyanopindolol	16 ± 5	26 ± 6	23 ± 4

experiments is shown in table 4.3. This again indicates that mesulergine, methysergide and ketanserin are the most effective antagonists.

4.7.3. The specificity of the serotonin antagonists

The results of the previous section demonstrate quite clearly that the antagonists inhibit the cyclic AMP and aldosterone response to serotonin. However, it is unclear if the effects observed are due to the presence of specific serotonin receptors in the zona glomerulosa or are caused by non-specific or toxic effects of the antagonists on the cells. In order to investigate this hypothesis the effects of each the antagonists were studied on the aldosterone response to angiotensin II, ACTH and potassium.

Figure 4.6 shows a typical aldosterone dose response to angiotensin II (0, 10^{-10}, 10^{-8} M) in the presence and absence of each of the serotonin antagonists (10^{-6} M). In this experiment mesulergine significantly inhibited basal secretion. However, the inhibitory effect of ketanserin and methysergide on basal secretion was less pronounced and non-significant. At 10^{-10} M angiotensin II, all of the antagonists significantly inhibited aldosterone secretion.

The combined % inhibition of each of the antagonists on the aldosterone response to angiotensin II from the means of three individual experiments is shown in table 4.4.

Cyclic AMP was not measured in this experiment as angiotensin II stimulation of aldosterone secretion is mediated by the phosphatidylinositol second messenger system and not the adenylate cyclase system.

Figure 4.7 shows a typical aldosterone dose response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of each of the serotonin antagonists (10^{-6} M). Although basal secretion was significantly inhibited by ketanserin, mesulergine and methysergide, the dose response curve was not significantly affected by the presence of any of the antagonists.

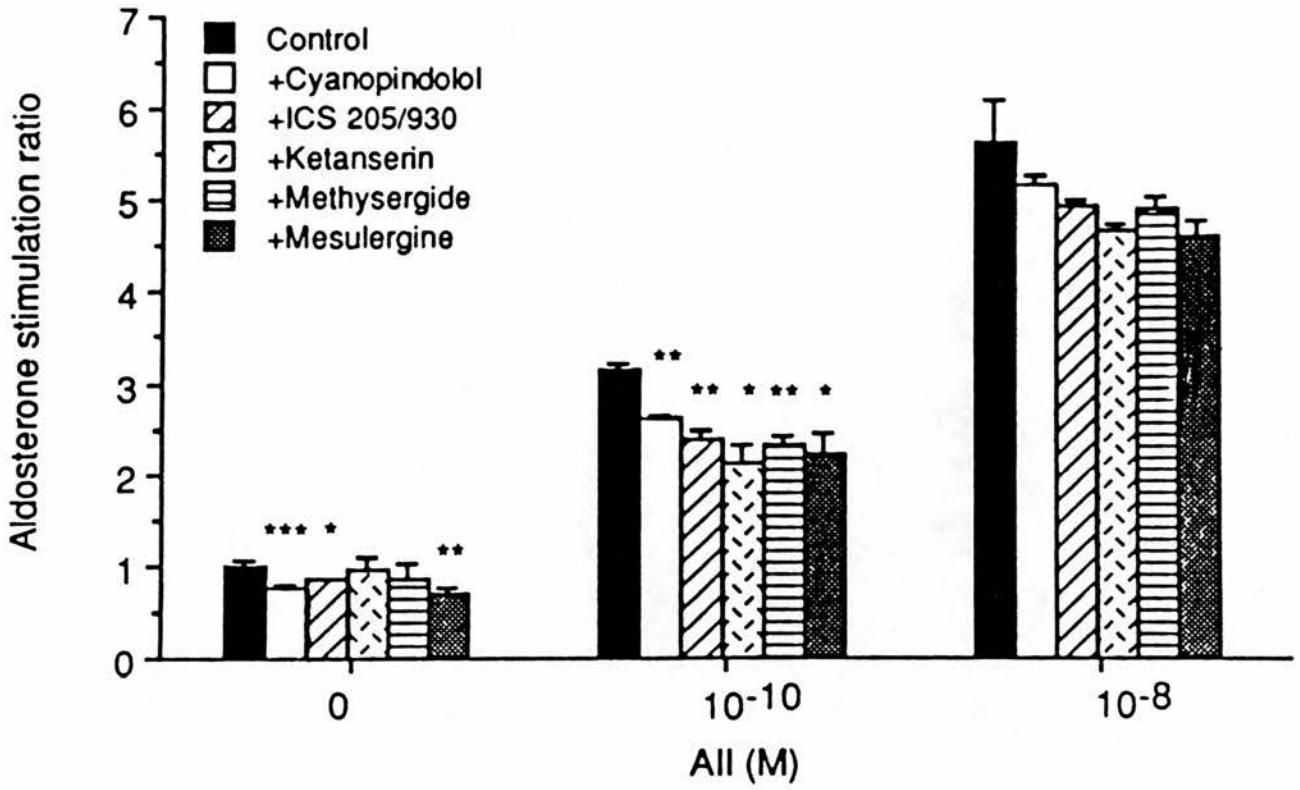


Figure 4.6.

The effect of serotonin antagonists (10^{-6}M) on the aldosterone dose response to angiotensin II.

* p<0.05 versus control
** p<0.01 versus control
*** p<0.001 versus control

Table 4.4.

The combined % inhibition ± SEM of each antagonist on the aldosterone response to angiotensin II from 3 experiments

	Angiotensin II Concentration (M)		
Antagonist	**0**	**10^{-10}**	**10^{-8}**
Mesulergine	20 ± 1	29 ± 7	21 ± 8
Methysergide	15 ± 0.2	23 ± 6	21 ± 4
Ketanserin	11 ± 0.5	25 ± 9	15 ± 3
ICS 205/930	10 ± 0.4	22 ± 6	10 ± 2
Cyanopindolol	17 ± 1	21 ± 6	17 ± 4

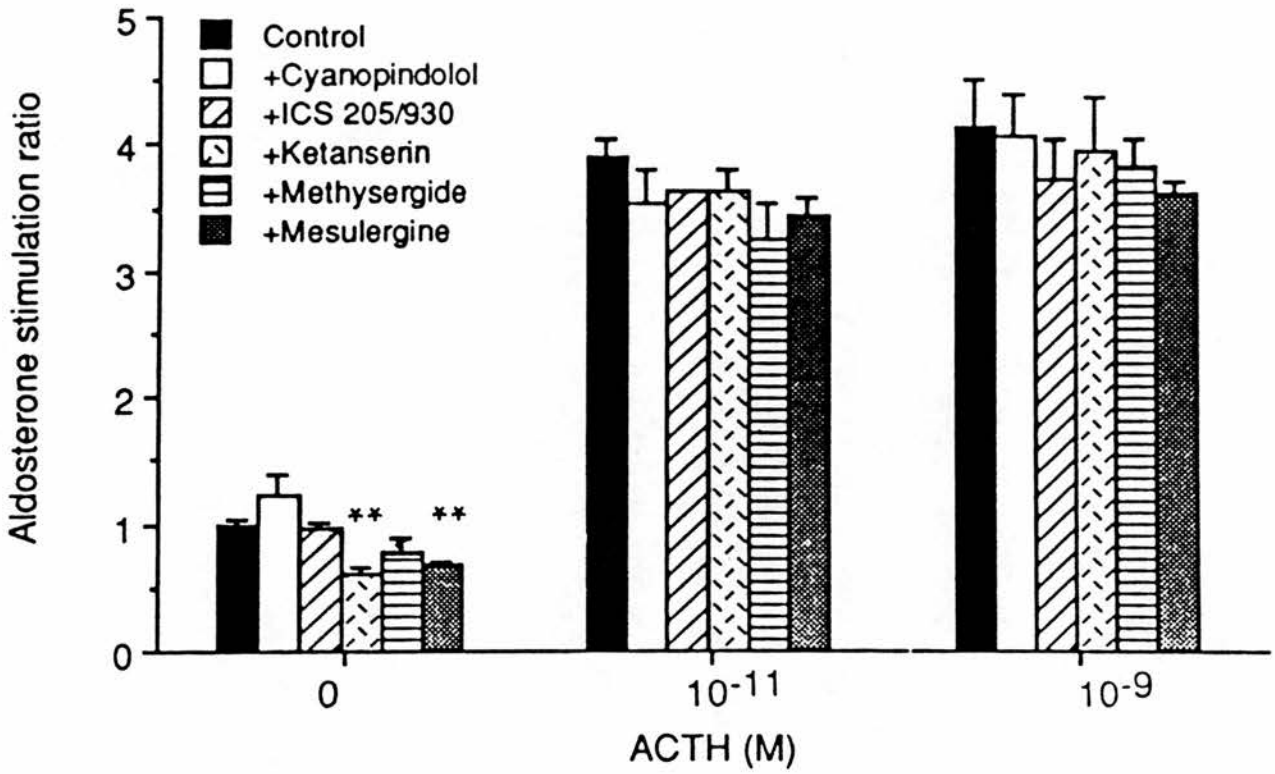


Figure 4.7.

The effect of serotonin antagonists (10^{-6}M) on the aldosterone dose response to ACTH.

* p<0.05 versus control
** p<0.01 versus control
*** p<0.001 versus control

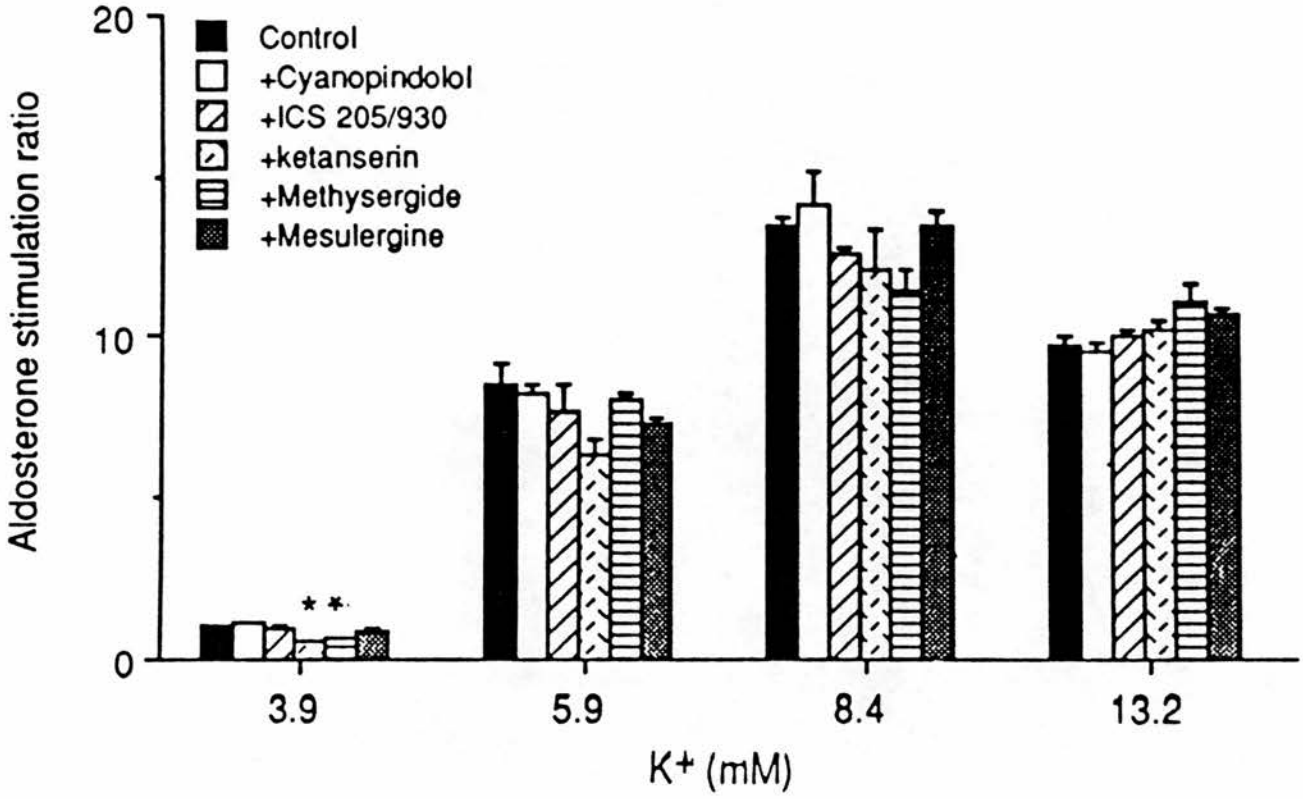


Figure 4.8.

The effect of serotonin antagonists (10^{-6}M) on the aldosterone dose response to potassium.

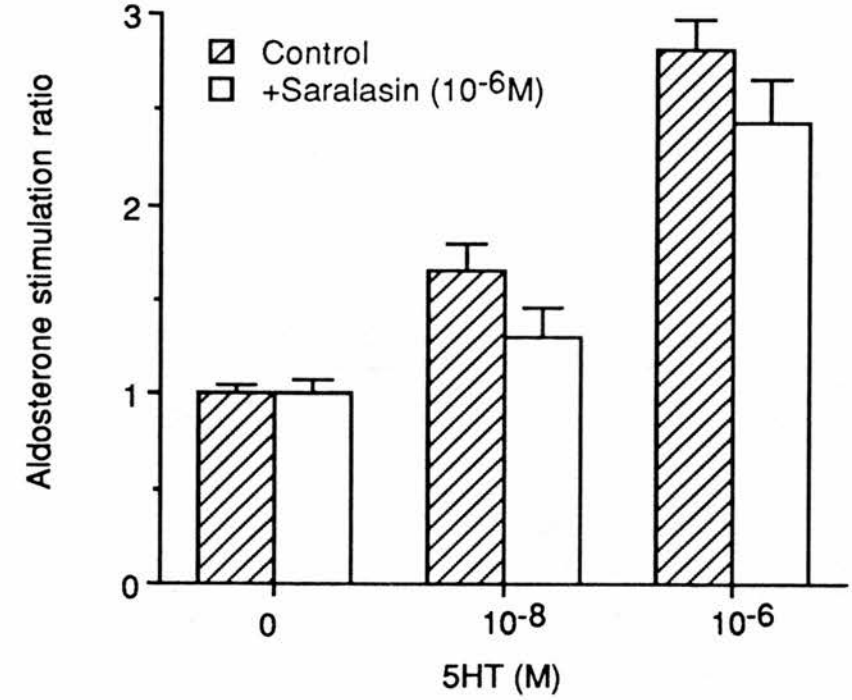
Figure 4.8 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4, 13.2 mM) in the presence and absence of each of the serotonin antagonists (10^{-6} M). Although basal aldosterone secretion was significantly inhibited by ketanserin and methysergide the dose response curve was not significantly affected by the presence of any of the antagonists.

4.7.4. The adrenal interaction of serotonin and angiotensin II

The results of the previous section demonstrate that the serotonin antagonists inhibit angiotensin II stimulated aldosterone secretion, particularly at low agonist concentration, but have no effect on the response to other stimuli. This suggests a possible interaction between angiotensin II and serotonin in the control of adrenal function. In order to investigate whether serotonin mediates its effects by acting at least partially on the angiotensin II receptor, the effects of the angiotensin II antagonist saralasin were studied on the aldosterone response to serotonin and angiotensin II as a control. In addition, the additive effects of serotonin and angiotensin II on steroid secretion were also studied.

Figure 4.9 shows a typical aldosterone dose response to angiotensin II (0, 10^{-10}, 10^{-8} M) or 5HT (0, 10^{-8}, 10^{-6} M) in the presence and absence of saralasin (10^{-6} M). The aldosterone dose response to angiotensin II was completely abolished in the presence of saralasin, although basal aldosterone secretion was not affected. The aldosterone response to serotonin was not affected by the presence of saralasin.

Figure 4.10 shows a typical aldosterone dose response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of angiotensin II (10^{-10}, 10^{-8} M). 10^{-10} M angiotensin II caused a significant increase in basal secretion as expected. However, it had no significant effect on 10^{-8} or 10^{-6} M serotoninergic stimulated secretion. 10^{-8} M angiotensin II significantly enhanced basal secretion and potentiated the effect of 10^{-8} M serotonin but not 10^{-6} M serotonin.



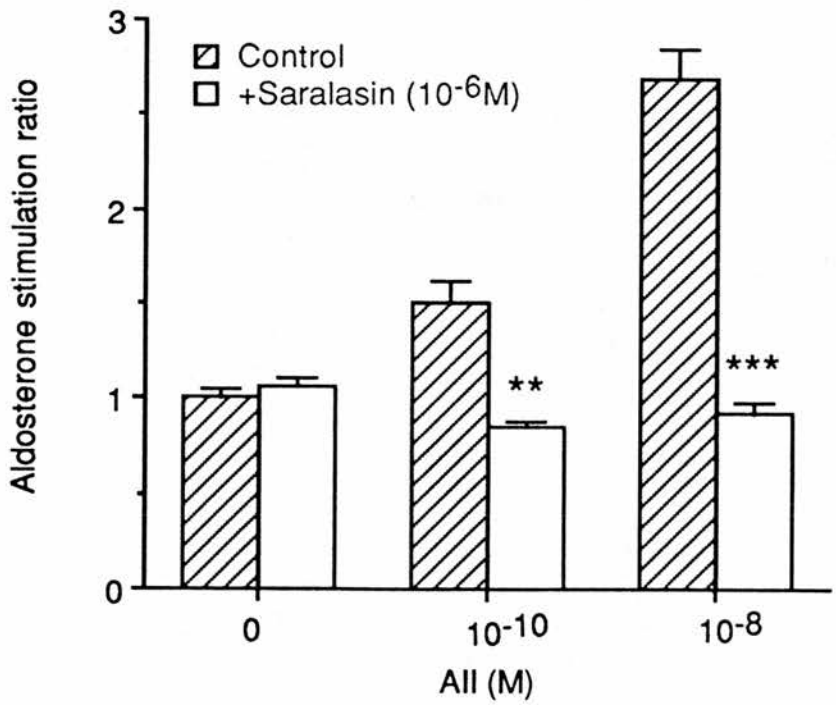


Figure 4.9. **The effect of saralasin on the aldosterone response to serotonin and angiotensin II.** (n=3)

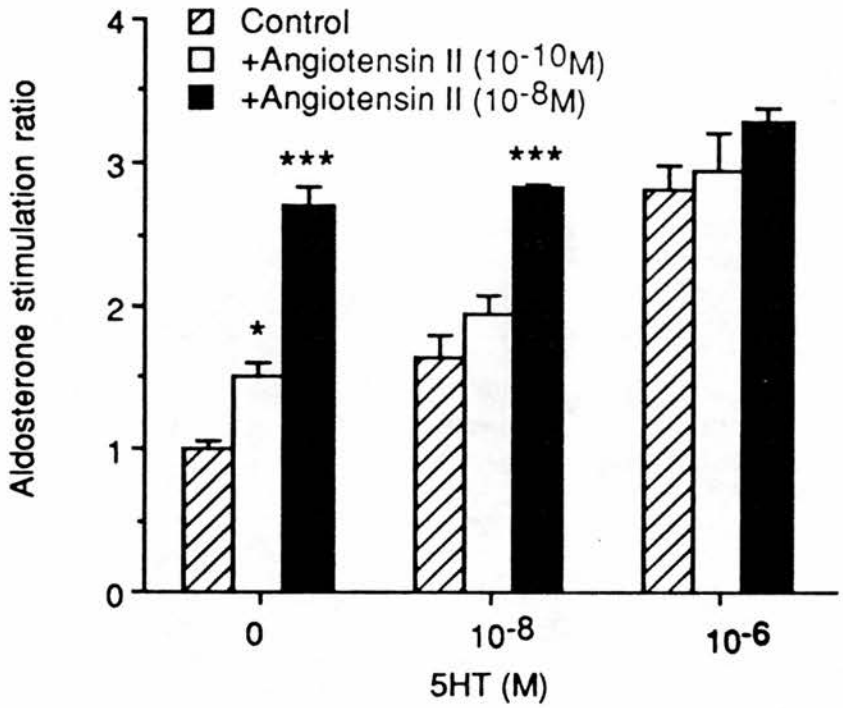


Figure 4.10. **The effect of serotonin, angiotensin II and serotonin plus angiotensin II on aldosterone secretion.** (n=3)

4.8. Discussion

The effects of a number of serotonin antagonists, which have a high affinity for a wide range of serotonin receptors, were studied on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium , in an attempt to characterise the serotonin receptor within the zona glomerulosa, in addition to assessing the specificity of the antagonists utilised. The lack of availability of a suitable radioactive derivative of serotonin make radioligand binding studies in adrenal cells extremely difficult; ^{3}H-5HT has a very low specific activity, whilst ^{125}I- 5HT is unstable and causes structural distortion of the serotonin molecule. These technical limitations necessitate the use of largely indirect pharmacological studies on the end steroid response to serotoninergic stimulation.

The results obtained from these studies confirm those of other groups that serotonin acts directly on the zona glomerulosa cells to increase aldosterone secretion *in vitro* (Müller and Zeigler 1968, Haning *et al* 1970, Tait *et al* 1972, Bing and Schulster 1977). Serotonin caused a 2.5 to 4 fold increase in aldosterone output, reaching maximal stimulation at approximately 10^{-6} M. The stimulatory capacity observed compares favourably with a number of groups but is slightly lower than the 8 fold increase shown by Tait *et al* 1972.

The introduction of ketanserin, methysergide and mesulergine, which have a high affinity for the $5HT_2$, $5HT_{1/2}$ and $5HT_{1c}$ receptors respectively, caused a significant inhibition of basal aldosterone secretion, although this effect was somewhat variable between experiments. No inhibition of basal secretion was observed with ICS 205/930 or cyanopindolol, which have a high affinity for the $5HT_3$ and $5HT_{1a/1b}$ receptors respectively. Basal inhibition has also been observed by a number of other groups for ketanserin, methysergide and metergoline (Müller and Zeigler 1968, Mendelsohn *et al* 1981, Matsuoka *et al* 1985). Due to the large inhibition of basal secretion the possibility of adrenal toxicity by ketanserin, methysergide and mesulergine was therefore considered. However, it was thought to be improbable, mainly because of their lack of inhibition on the aldosterone dose

response to either ACTH or potassium. The lack of inhibition of the antagonists on the aldosterone dose response to ACTH has been confirmed for ketanserin, methysergide and cyproheptadine by other groups (Müller and Zeigler 1968, Matsuoka *et al* 1985), although in contrast, other workers have reported inhibition of the aldosterone response to potassium and ACTH with ketanserin (Rocco *et al* 1986). A more plausible explanation for the basal inhibition is the presence of endogenous serotonin within the adrenal itself as has been previously reported at least in the medulla (Verhofstadt and Jonsson 1983, Holzwarth *et al* 1984). However, although radiolabelling studies have shown the rapid uptake of serotonin and its metabolism to 5HIAA, there is very little conclusive evidence of serotonin storage within the capsular tissue (Trost and Müller 1976). If endogenous serotonin is present it may occupy serotonin receptors and exert a tonic stimulatory influence on aldosterone production, an effect which is negated by the introduction of the antagonists which displace the endogenous serotonin. Endogenous serotonin concentration may vary in each cell preparation, and the differing degrees of basal inhibition for the same antagonist between experiments may be indicative of this concentration. It is also feasible this concentration of serotonin may in some way be related to stress levels in the animals preceding cervical dislocation. Stress facilitates the release of ACTH from the pituitary gland resulting in increased adrenal perfusion by the blood. Serotonin which is transported by the platelets may be released locally at the adrenal and bind to the serotonin recognition sites within the zona glomerulosa. Therefore, the more stressed an animal becomes the more serotonin could be released, by a mechanism other than the blood clotting cascade.

Alternatively, the serotonin antagonists may exhibit partial dopaminergic agonist properties which would enhance the tonic inhibitory influence of dopamine on aldosterone secretion, resulting in decreased basal output (Carey *et al* 1979). High affinities of serotonin antagonists for dopamine receptors have been reported by Leysen *et al* 1981, who showed spiperone and haloperidol were 7 and 40 times more potent at dopamine than at $5HT_2$ receptors. A high affinity for the dopamine receptor was also reported for methysergide.

However, only very high concentrations (>10^{-5} M) of dopamine have been shown to inhibit basal aldosterone secretion and the effect is only moderate, therefore it is unlikely that a lower dose of a serotonin antagonist would act on dopamine receptors (Aguilera and Catt 1984).

The stimulatory capacity of serotonin on aldosterone output was blocked to different degrees by all the antagonists we tested. Based purely on the % inhibition from 3 combined experiments, the order of effectiveness of the antagonists was mesulergine > methysergide > ketanserin > ICS 205/930 > cyanopindolol, the latter two showing only minor inhibition. Serotonin also caused a significant increase in cyclic AMP secretion, but only at or above 10^{-8} M. 10^{-9} M serotonin failed to stimulate cyclic AMP, although steroid secretion was enhanced. This apparent dissociation between second messenger and end steroid response has also been observed by other groups and is similar to the results obtained with ACTH (Beall *et al* 1972, Albano *et al* 1974, Fujita *et al* 1979). The cyclic AMP response was also inhibited to varying degrees by all the antagonists tested, and these showed a similar order of potency for inhibition of cyclic AMP as they did for aldosterone.

The results suggest that *in vitro*, the aldosterone response to serotonin is mediated by specific serotonin receptors located in the zona glomerulosa and these are closely coupled to the adenylate cyclase second messenger system. However, identifying the specific receptor(s) involved is a more difficult task, even although a wide range of antagonists was employed.

Before the heterogeneity of the $5HT_1$ receptor was discovered, methysergide was originally classified as a $5HT_1$ receptor antagonist which also displayed a relatively high affinity for the type-2 binding site. Ketanserin and the more recently developed mesulergine on the other hand were considered to be selective for the $5HT_2$ and $5HT_{1C}$ sites respectively. Therefore, the particularly potent inhibitory properties of mesulergine and methysergide suggest that the aldosterone response to serotonin is predominantly a $5HT_1$ mediated effect, and this is consistent with the cyclic AMP data and the fact that $5HT_1$ receptors are coupled to

the adenylate cyclase second messenger system (Enjalbert *et al* 1978, Peroutka *et al* 1981).

However, a recent review by Hoyer which examined the binding affinities of a wide range of serotonin antagonists for each of the serotonin receptors, clearly shows that some of the antagonists may not be as specific for one receptor as originally believed (Hoyer 1988a). The pK_D values reported by Hoyer of ketanserin, methysergide, mesulergine, ICS 205/930 and cyanopindolol for each of the serotonin receptors are shown in table 4.5. The data shows clearly that those antagonists which display a high affinity for the $5HT_{1c}$ binding site, e.g mesulergine also have an equally high affinity for the type-2 binding site. Similarly, methysergide displays equally high affinity for the $5HT_{1c}$, $5HT_2$ site and $5HT_{1d}$ binding sites. In contrast, cyanopindolol displays very low affinity for the $5HT_{1c}$ and $5HT_2$ sites, but does have a high affinitiy for both the $5HT_{1b}$ and $5HT_{1a}$ binding sites. The data also reveals that although ketanserin is one of the most selective antagonists available, it does display moderate affinity for the $5HT_{1c}$ site, which is approximately 100 fold less than the magnitude of its affinity for the $5HT_2$ site. ICS 205/930 remains selective for the $5HT_3$ site.

Considering these facts together with the results obtained in the zona glomerulosa, it seems clear from the weak inhibitory effects of cyanopindolol and ICS 205/930, that the $5HT_{1a}$, $5HT_{1b}$ and $5HT_3$ receptors play very little, if any role, in mediating the aldosterone response to serotonin. Similarly, the $5HT_{1d}$ receptor can be eliminated as methysergide is the only antagonist which displays appreciable affinity for this receptor. Therefore, it seems likely that the potent inhibitory properties of mesulergine, methysergide and ketanserin are due to the presence of $5HT_2$ and / or $5HT_{1c}$ receptors within the zona glomerulosa. However, which receptor predominates is less clear. If it is the type-2 receptor then ketanserin, which has the highest affinity for this receptor, would be expected to produce the greatest inhibititory effect and this was not the case. If it is the $5HT_{1c}$ receptor the order of effectiveness of the drugs is consistent with their individual affinities for the $5HT_{1c}$ site

Table 4.5.

The affinity values of the antagonists for the $5HT_1$ and $5HT_2$ receptors (pK_D values -log mol/l).

DRUG	$5HT_{1a}$	$5HT_{1b}$	$5HT_{1c}$	$5HT_{1d}$	$5HT_2$
Mesulergine	6.23	4.88	8.79	5.20	8.42
Methysergide	7.63	5.82	8.61	8.42	8.57
Ketanserin	5.86	5.72	7.01	6.00	8.86
Cyanopindolol	8.27	8.28	4.44	6.85	4.53
ICS 205/930	4.70	4.35	4.59	------	5.28

(Taken from Hoyer 1988)

reported by Hoyer and it seems this is the most likely. This theory was supported by a more detailed pharmacological profile of the adrenal serotonin receptor, carried out by a student in the department (LA Bishop 1989, BSc Honours project, Department of Pharmacology, University of Edinburgh). By using varying concentrations of serotonin and each antagonist, the dose-ratio was calculated for each concentration and Schild plots were set up. Utilisation of the Schild equation provided an estimation of the dissociation constant for each of the antagonists for the serotonin binding site (Arunlakshana and Schild 1959). The results obtained are illustrated in table 4.6, and they show that the $5HT_{1c}$ antagonist mesulergine has the highest binding affinity. Although this is indicative of the presence of the $5HT_{1c}$ receptor, a more definitive demonstration of its specific involvement, rather than a $5HT_2$ site, must await the development of a more selective $5HT_{1c}$ receptor antagonist.

It is interesting that current evidence from studies in other tissues, indicates that the $5HT_{1c}$ binding site bears striking similarities to the $5HT_2$ site both pharmacologically in that it exhibits high affinity for so-called $5HT_2$ selective antagonists and functionally in that its signal transduction mechanism is not via the adenylate cyclase system as is usual for $5HT_1$ receptors, but via the phosphatidylinositol (PI) system normally associated with the type-2 receptors (Hoyer 1988b). However, the increased cyclic AMP levels observed during serotoninergic stimulation of aldosterone secretion both by this laboratory and a number of other groups (Tait *et al* 1974, Albano *et al* 1974, Fujita *et al* 1979), suggest that in the adrenal gland the $5HT_2$ and/or $5HT_{1c}$ receptors are coupled to the adenylate cyclase system and not the PI system. This supports the view of other workers (Matsuoka *et al* 1985) and ultimately indicates that the same serotonin receptors may be coupled to different G-proteins in different tissues. A more detailed study of the signal transduction system coupled to serotoninergic stimulation is carried out in the next chapter.

It is also conceivable that in addition to the serotonin receptor another receptor type

Table 4.6.

The estimated pK_D values of the serotonin antagonists for the zona glomerulosa serotonin receptor.

DRUG	pK_D
Ketanserin	6.01
Mesulergine	7.63
Methysergide	7.56
Cyanopindolol	5.73
ICS 205/930	5.40

(Taken from Lisa Bishop's honours project)

could be involved in the adrenal response to serotonin. The α–adrenergic receptors have been previously reported to partly mediate the anti-hypertensive action of ketanserin at high concentrations (Fozard 1982). However, their involvement has been eliminated by previous studies in our laboratories which demonstated that neither prazosin, a selective α_1 antagonist, nor phentolamine, a non selective α–antagonist, inhibited the aldosterone response to serotonin in rat adrenal zona glomerulosa cells (Williams *et al* 1984).

Although the antagonists utilised do not inhibit the aldosterone response to ACTH and potassium, thus excluding adrenal toxicity, they do however inhibit the aldosterone response to angiotensin II, particularly at low agonist concentration. Once again mesulergine, methysergide and ketanserin appear to be the most effective. This inhibitory effect of the serotonin antagonists on angiotensin II stimulated aldosterone secretion has been reported by other workers at least for ketanserin and cyproheptadine (Matsuoka *et al* 1985, Rocco *et al* 1986). In addition, radioligand binding studies carried out in this department using ^{125}I-angiotensin II have shown conclusively that ketanserin and methysergide inhibit the binding of angiotensin II to rat zona glomerulosa cells (CJ Waugh *et al*, unpublished data).

The inhibitory effect of the serotonin antagonists on angiotensin II stimulated aldosterone secretion may be explained in a number of ways. Firstly, the drugs may be toxic and affect a number of important cellular processes non-specifically. Secondly, that serotonin increases aldosterone output by acting at least partially on the angiotensin II receptor, thirdly that the effects are due to the dopaminergic agonist properties of the drugs used, or finally that local serotonin and its receptor exert a modulatory role on the angiotensin II receptor and hence the aldosterone response.

The first possibility has been excluded at least partly, in that the intact cellular response to ACTH and potassium in the presence of the antagonists makes adrenal toxicity very unlikely.

The second possibility that serotonin stimulates aldosterone by acting directly on the angiotensin II receptor seems unlikely, not only as the compounds are so structurally dissimilar

but also because the presence of saralasin (10^{-6} M), a specific angiotensin II receptor antagonist, does not inhibit the aldosterone response to serotonin *in vitro*, although the response to angiotensin II is completely abolished. A similar result was found by Mendelsohn and Kachel 1981, who demonstrated that the stimulatory effect of serum containing serotonin on aldosterone secretion could not be blocked by saralasin, although the effect of angiotensin II was completely abolished. The additivity experiments were also designed to show that serotonin and angiotensin II act on different receptors. However, there is no additive effect in the true sense that if each stimulus caused a 2 fold increase in steroid secretion, then together they should cause a 4 fold increase, if they act on separate receptors. There is however some degree of potentiation, particularly with high doses of one stimulus and low doses of the other. The lack of additivity suggests that each stimulus may not act completely independently and may interact with each other.

The third hypothesis that the inhibitory properties of the serotonin antagonists on the aldosterone response to angiotensin II may be due to their dopaminergic agonist properties is a feasible explanation. Dopamine has been shown to inhibit angiotensin II stimulated aldosterone secretion both *in vivo* and *in vitro* (McKenna *et al* 1979, Edwards *et al* 1980a, Aguilera *et al* 1984). Similarly, metoclopramide, a dopamine antagonist, potentiates the aldosterone response to angiotensin II (Edwards *et al* 1980a). However, these effects are only observed at very high doses (>10^{-5} M) of dopamine, whereas the effects here are observed with 10^{-6} M antagonist.

However, the final possibility that serotonin and its receptor can interact with the angiotensin II receptor and exert a modulatory role seems the most likely. Therefore, blocking the serotonin receptor with the various antagonists decreases the adrenal responsiveness to angiotensin II. This supposed synergistic relationship between angiotensin II and serotonin is not a new theory and many groups have reported similar effects in a wide range of tissues. Serotonin potentiates the vasocontrictive action of angiotensin II and a number of other substances such as catecholamines and prostaglandins (Van Neuten *et al* 1982a, Weiner *et al*

1987). Conversely, it is possible that angiotensin II may exhibit a modulatory effect on the stimulatory properties of serotonin, as behavioural studies in rats have shown angiotensin II increases serotonin and tryptophan induced hyposensitivity to painful stimuli and also yawning which is a serotonin dependent process, and this potentiation could be blocked by the serotonin antagonists mianserin and ketanserin. The enhancing action of angiotensin II may be mediated by an increase in number and/or sensitivity of post-synaptic serotonin receptors as it does not result from altered synthesis, release or uptake of serotonin (Braszko *et al* 1985). Similarly, other studies using isolated cells either statically or in a perfusion system, have shown that a sodium deplete diet augments the *in vitro* aldosterone response to serotonin, whilst a sodium replete diet diminishes the response (Al-Dujaili *et al* 1982, Shaikh *et al* 1986). However, this interaction may not specifically relate to serotonin alone, as the sensitivity of the response to other stimuli are similarly affected. Diverging somewhat from steroidogenesis but strenghthening the argument for an interaction between angiotensin II and serotonin, it has recently been found that the mas-oncogene encodes an angiotensin II receptor, whilst the $5HT_{1c}$ receptor acts under certain circumstances as a proto-oncogene (Jackson *et al* 1988, Julius *et al* 1989).

The pharmacological profile and putative identification of a second messenger system suggests the existence of specific serotonin receptors within the zona glomerulosa. However, conclusive evidence requires a more detailed pharmacological profile and molecular experiments, probing the adrenal cortex for the presence of unique amino acid sequences encoding the serotonin receptor(s). The $5HT_{1a}$, $5HT_{1c}$ and $5HT_2$ receptors from other tissues have already been cloned and sequenced (Julius *et al* 1988, Fargin *et al* 1988, Pritchett *et al* 1988). The serotonin receptor type(s) which mediates the effect of serotonin on aldosterone secretion cannot be fully classified with the available antagonists, although it seems likely that the $5HT_{1c}$ and /or $5HT_2$ receptor types predominate and in contrast to other tissues, in the zona glomerulosa these appear to be coupled to the adenylate cyclase signal

transduction system.

In addition, serotonin may also modulate the action of angiotensin II, as it does in other tissues. This modulatory effect may be responsible for increasing adrenal responsiveness to angiotensin II *in vivo*. This interaction may be an important physiological regulatory component of mineralocorticoid secretion and blood pressure control and the nature of this interaction and precise characterisation of the receptors involved requires further investigation. However, the ability of specific serotonin antagonists to inhibit the aldosterone response to serotonin and angiotensin II could find useful therapeutic application in the future treatment of disorders associated with altered adrenal function.

Chapter Five

The Mechanism of Action of Serotonin *In Vitro*.

5.1. Introduction

Although the stimulatory effect of serotonin on aldosterone secretion *in vitro* is well documented, the mechanism by which the effect is mediated remains unclear.

The results from the previous chapter indicate that specific serotonin receptors exist within the zona glomerulosa and these appear primarily to be of the $5HT_{1C}$ and/or $5HT_2$ classes. However, in direct contrast to the central nervous system and periphery, where they are coupled to the PI system, in the adrenal gland they appear to be coupled to the adenylate cyclase second messenger system. In other well characterised models acting via adenylate cyclase e.g ACTH, the enzymatic changes are closely coupled to ionic changes, particularly alterations in Ca^{2+} transport.

Although a number of groups have confirmed the involvement of adenylate cyclase in the action of serotonin on the adrenal gland, controversy surrounds the role of the PI system, and only a limited number of studies have focussed on the synarchic role of Ca^{2+}.

This chapter aims to study the intracellular mechanisms, in particular the role of adenylate cyclase and Ca^{2+}, which mediate the aldosterone response to serotonin following the binding to its zona glomerulosa receptor and compare these with the already well characterised mechanisms of action of angiotensin II, ACTH and potassium.

5.2. Aims of study

1. To study the involvement of the adenylate cyclase and the PI second messenger system in the action of serotonin on the adrenal and compare this with the well characterised action of angiotensin II.
2. To study the role of calcium as a mediator of the steroid response to serotonin by utilising calcium antagonists which block either extra or intracellular movement of calcium
3. To study the calcium requirement directly, by carrying out $^{45}CaCl_2$ influx studies and comparing any changes with those observed with angiotensin II.

5.3. Materials and Methods

The isolation of zona glomerulosa cells, static incubations, radioimmunoassays, percoll density gradient cell purification and calcium influx methodology has been previously described in chapter 3, sections 3.2 to 3.3 and 3.7. Measurement of the components of the PI system has been previously described by Bird *et al* 1987.

5.4. Graphical Illustration

As in chapter 4, with the exception of the radiolabelled calcium influx experiments, the graphs illustrated are single experiments incubated in triplicate, and are representative of the general trends observed in each study. Each study was repeated at least once and on most occasions twice. Aldosterone secretion is expressed as a stimulation ratio $\pm$ SEM in relation to the basal output of the control sample which has been given a value of 1. The mean basal aldosterone secretion from 10 individual experiments was 1.000 $\pm$ 0.100 nM. The mean basal cyclic AMP secretion from 6 individual experiments was 0.449 $\pm$ 0.111 nM.

For the radiolabelled Ca^{2+} uptake studies, the increase in intracellular radiolabelled calcium is expressed as a % of the total number of counts added to each incubation at the beginning of the experiment i.e 5 μ Ci. The graphs are derived from pooling the values from a number of individual experiments.

5.5. Statistical Analysis

Statistical significance was calculated using Student's t-test for unpaired samples. A p value of <0.05 was considered significant. NS indicates non-significance. The p values in the steroid output studies are illustrated predominantly in the figures, though on some occasions in the text. The p values for the $^{45}Ca^{2+}$ influx studies are quoted in the text. *, ** and *** indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

5.6. Results

5.6.1. The effect of serotonin, angiotensin II, ACTH and potassium on aldosterone and cyclic AMP secretion.

This initial preliminary study illustrates the already well characterised changes in cyclic AMP and aldosterone secretion following stimulation with serotonin, angiotensin II, ACTH and potassium.

Figure 5.1 shows a typical aldosterone dose response to serotonin (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M) in the rat zona glomerulosa cell preparation used routinely throughout these studies. 10^{-8} M serotonin was sufficient to cause a significant increase in aldosterone secretion. Maximal stimulation was acheived by 10^{-6} M serotonin which caused a 3 fold increase in steroid output. In subsequent experiments only 2 concentrations of serotonin were studied; 10^{-8} and 10^{-6} M.

Figure 5.2 shows the corresponding cyclic AMP response to serotonin. Cyclic AMP output was not significantly increased at 10^{-8} M serotonin, although aldosterone secretion was elevated. 10^{-7} M serotonin caused a significant increase in cyclic AMP secretion and 10^{-6} M serotonin, which caused maximal steroidogenesis, caused a 3 fold increase in cyclic AMP.

Figure 5.3 shows a typical aldosterone dose response to angiotensin II (0, 10^{-10}, 10^{-8} M). Angiotensin II caused a significant dose-dependent stimulation of aldosterone secretion.

Figure 5.4 shows the corresponding cyclic AMP dose response to angiotensin II (0, 10^{-10}, 10^{-8} M). Angiotensin II did not cause any significant increase in cyclic AMP secretion at any concentration.

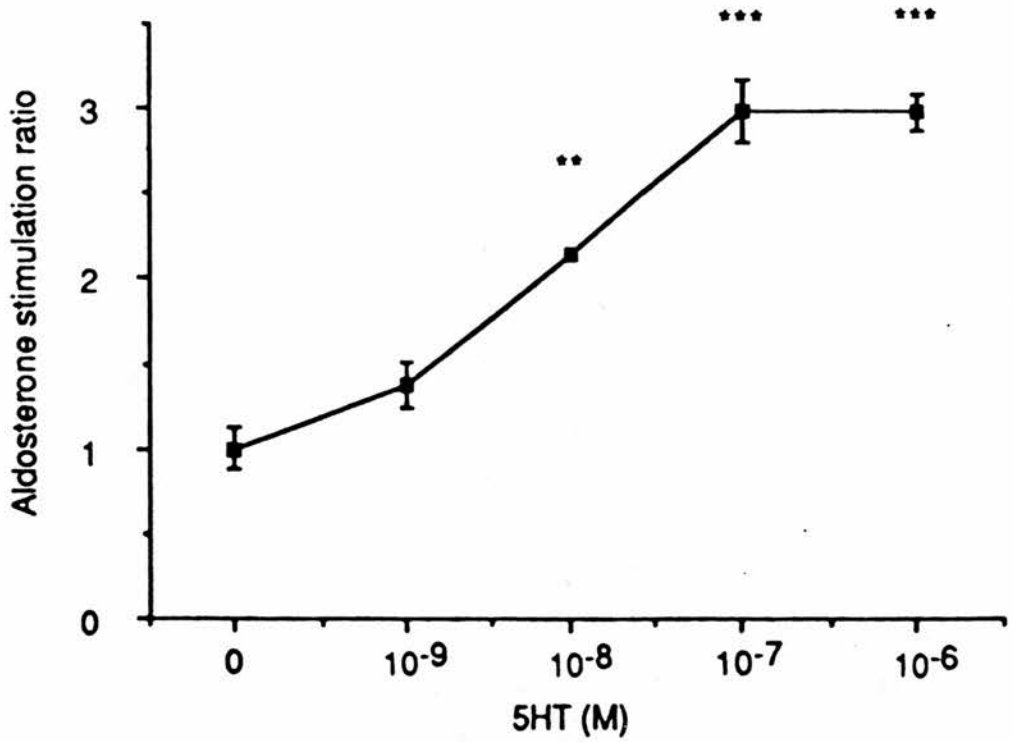


Figure 5.1. **A typical aldosterone response to serotonin.** (n=3)

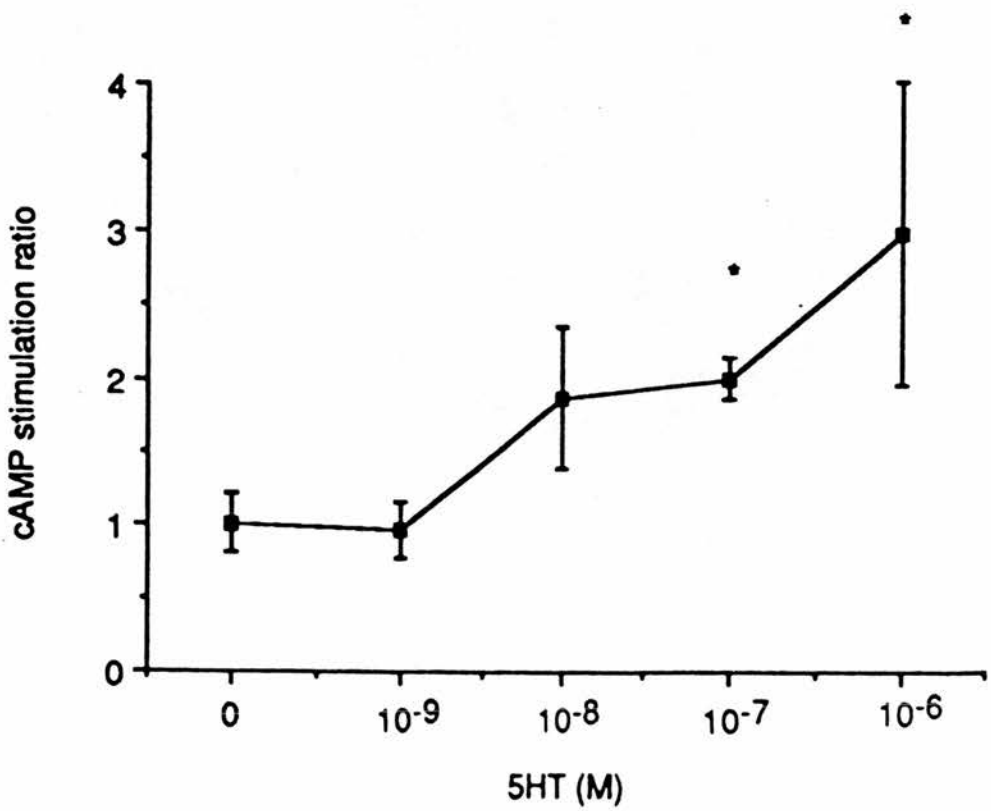


Figure 5.2. **A typical cyclic AMP response to serotonin.** (n=3)

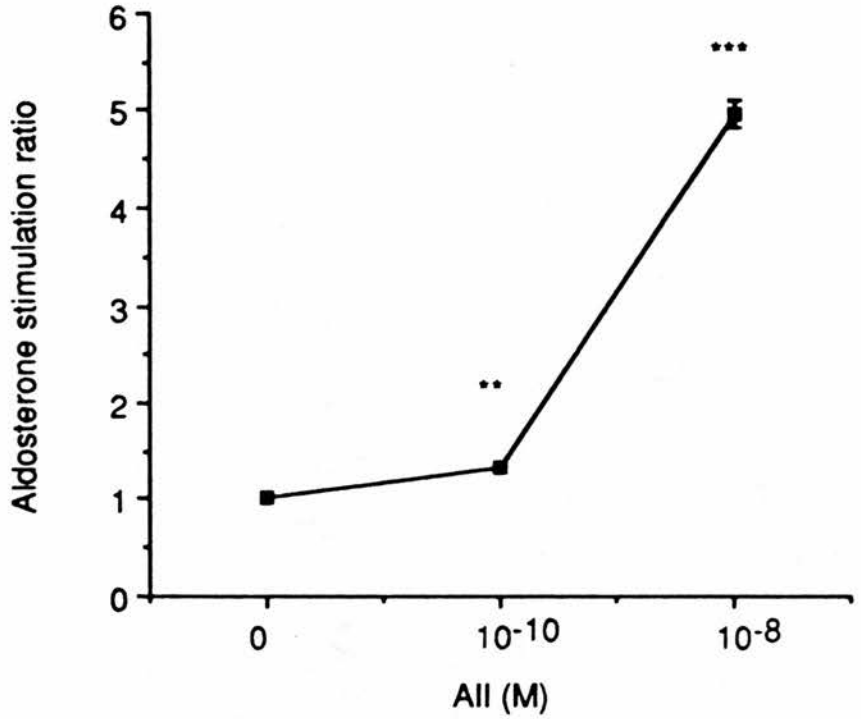


Figure 5.3. **A typical aldosterone response to angiotensin II.** (n=3)

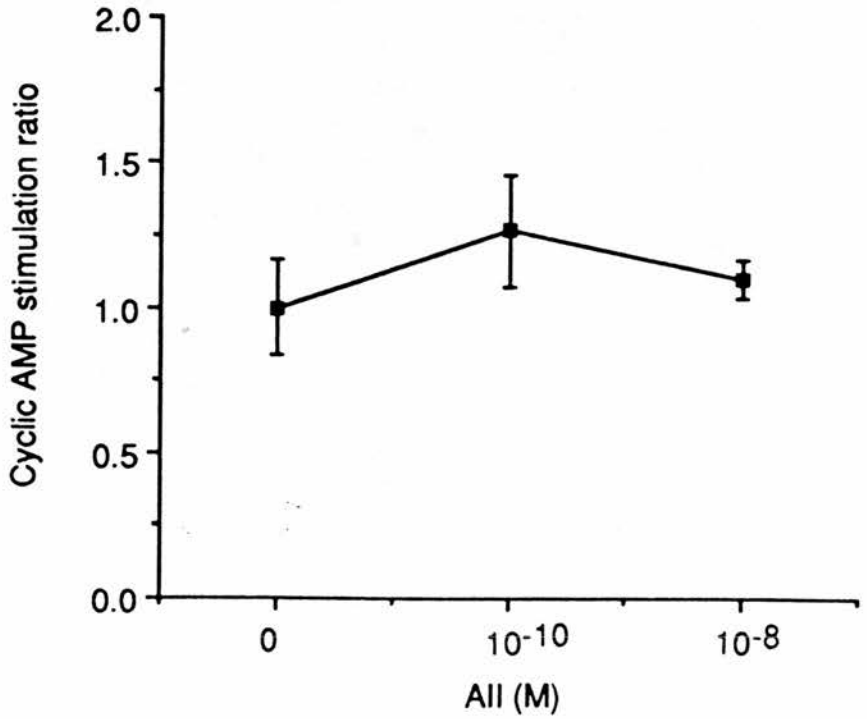


Figure 5.4. **A typical cyclic AMP response to angiotensin II.** (n=3)

Figure 5.5 shows a typical aldosterone dose response to ACTH (0, 10^{-11}, 10^{-9} M). ACTH caused a significant dose-dependent stimulation of aldosterone secretion.

Figure 5.6 shows the corresponding cyclic AMP dose response to ACTH (0, 10^{-11}, 10^{-9} M). ACTH stimulated cyclic AMP secretion, although the effect was only significant at 10^{-9} M. 10^{-11} M did not significantly increase cyclic AMP secretion, even although steroidogenesis was enhanced.

Figure 5.7 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4, 13.2 mM). 5.9 and 8.4 mM potassium caused a significant dose-dependent increase in aldosterone secretion. Compared with 8.4 mM, 13.2 mM potassium caused a decrease in aldosterone secretion, however the secretion was still significantly increased compared to the control group.

Figure 5.8 shows the corresponding cyclic AMP response to potassium (3.9, 5.9, 8.4, 13.2 mM). All 3 concentrations of potassium caused a significant increase in cyclic AMP secretion. This followed a similar secretory pattern to that of aldosterone, although the effect was much smaller.

5.6.2. The effect of serotonin and angiotensin II on various components of the phosphatidylinositol system.

The results of the previous study show clearly that, with the exception of angiotensin II, all the secretagogues increase secretion of the second messenger cyclic AMP. Angiotensin II is already known to act through th PI system. Therefore, the effect of angiotensin II, which acted as a positive control, on lipid, inositol and inositol phosphate content of zona glomerulosa cells was studied and compared with that of serotonin, which is known to be coupled to the PI system at least within the vascular system, through $5HT_2$ receptors. This

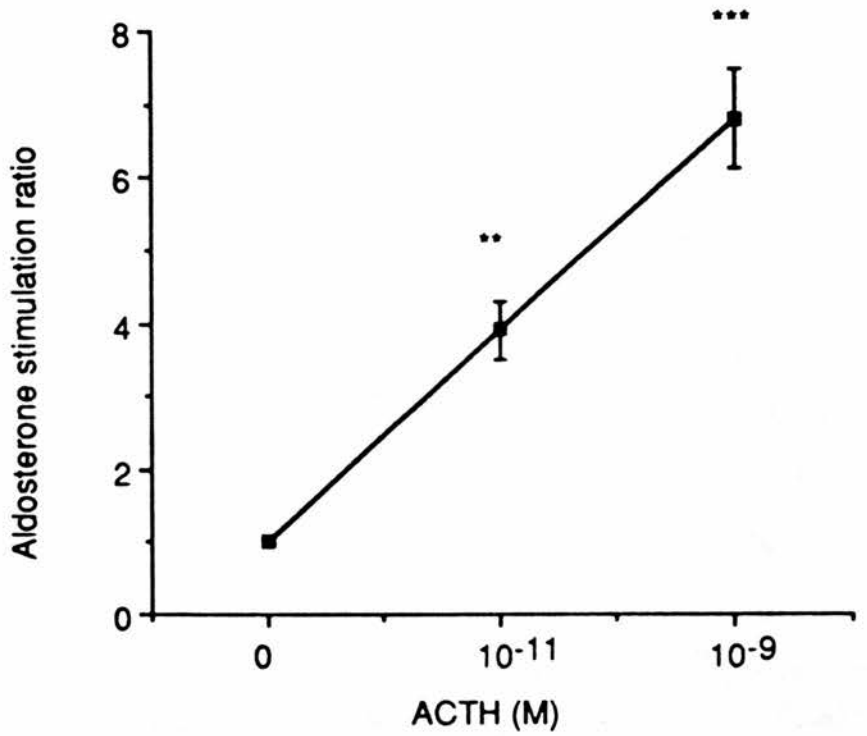


Figure 5.5. **A typical aldosterone response to ACTH.** (n=3)

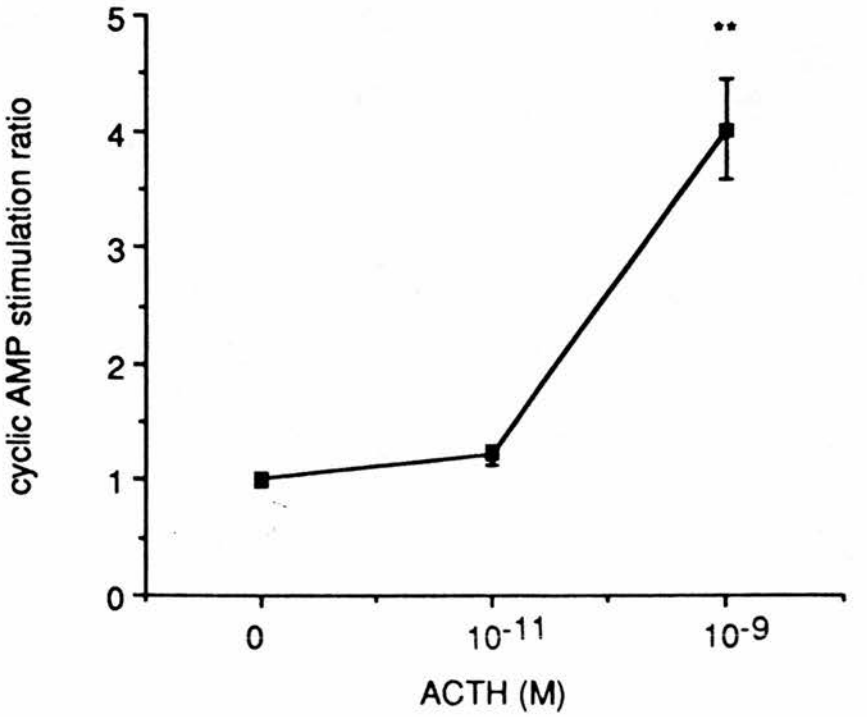


Figure 5.6. **A typical cyclic AMP response to ACTH.** (n=3)

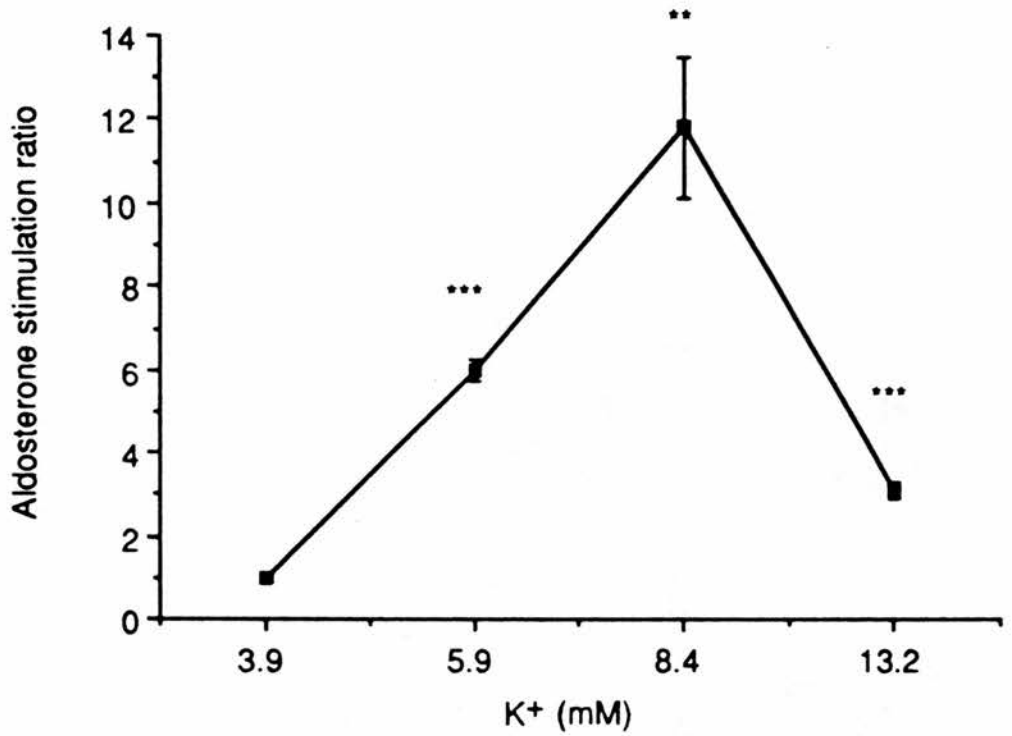


Figure 5.7. **A typical aldosterone response to potassium.** (n=3)

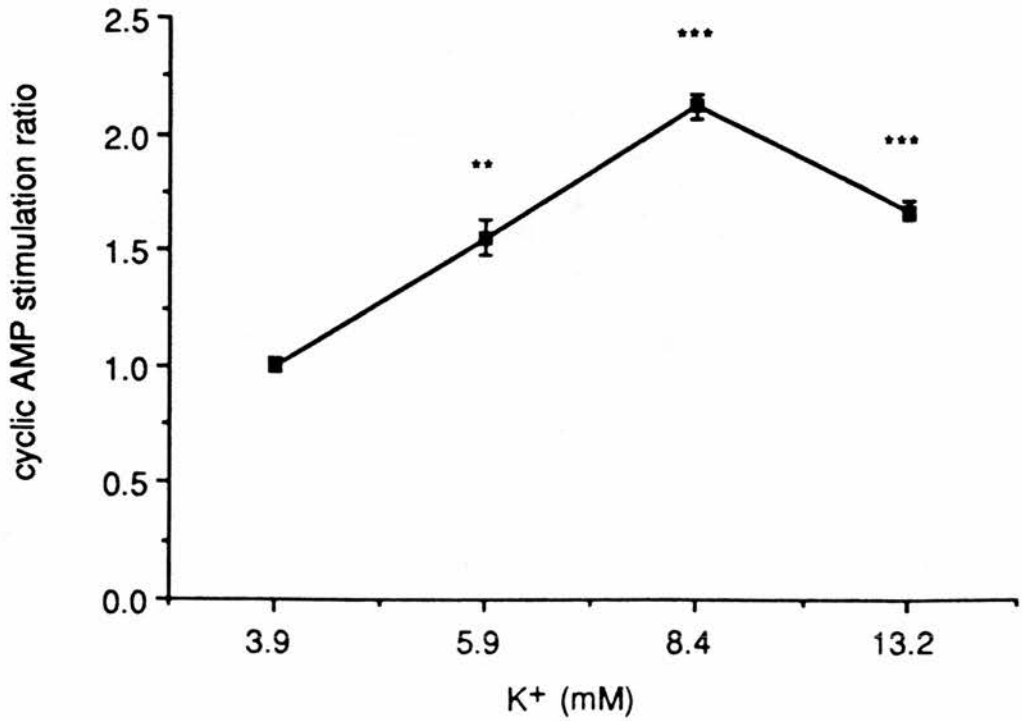


Figure 5.8. **A typical cyclic AMP response to potassium.** (n=3)

data was kindly provided by Dr I Bird of the Royal Infirmary, Edinburgh. The content of lipid, inositol phosphate and inositol within the cells following stimulation is given as a % value compared to the unstimulated control groups which have been given a value of 100%.

Figure 5.9 shows the lipid content of isolated zona glomerulosa cells following stimulation with angiotensin II (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M) or serotonin (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M). Angiotensin II caused a significant dose-dependent decrease in the lipid content of the cells, which decreased from 100 $\pm$ 2.6 % in the unstimulated control group to 91.1 $\pm$ 2.6, 65.4 $\pm$ 2.1, 39.6 $\pm$ 1.8 and 34.1 $\pm$ 1.3 % at 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M respectively. Serotonin showed no significant effect at any concentration.

Figure 5.10 shows the inositol phosphate (IPn) content of isolated zona glomerulosa cells following stimulation with angiotensin II (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M) or serotonin (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M). Angiotensin II caused a significant dose-dependent increase in IPn content of the cells, which increased from 100 $\pm$ 12.2 % to 173 $\pm$ 5.2, 392 $\pm$ 9.5, 623 $\pm$ 31.1 and 710 $\pm$ 66.5 % at 10 $^{-11}$, 10^{-10}, 10^{-9} and 10^{-8} M respectively. Serotonin showed no significant effect at any concentration.

Figure 5.11 shows the inositol (Ins) content of isolated zona glomerulosa cells following stimulation with angiotensin II (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M) or serotonin (0, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M). With the exception of 10^{-9} M angiotensin II, which caused a significant increase from 100 $\pm$ 1.9 to 113 $\pm$ 2.6 %, no other concentration of angiotensin II nor serotonin showed any significant effect on Ins content.

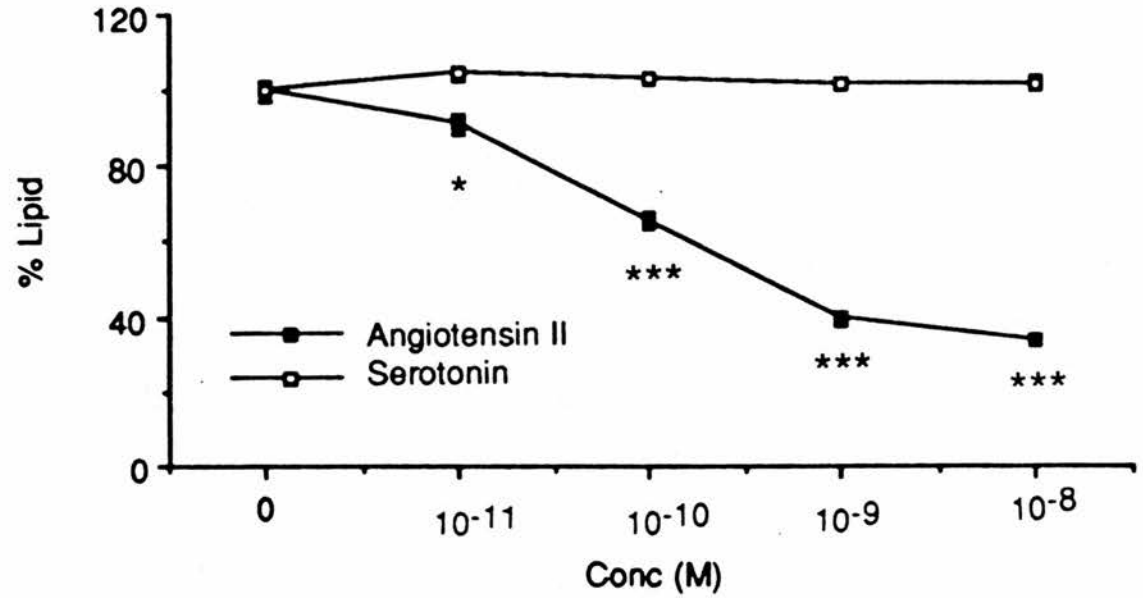


Figure 5.9. **The effect of angiotensin II and serotonin on cellular lipid content** (n=3)

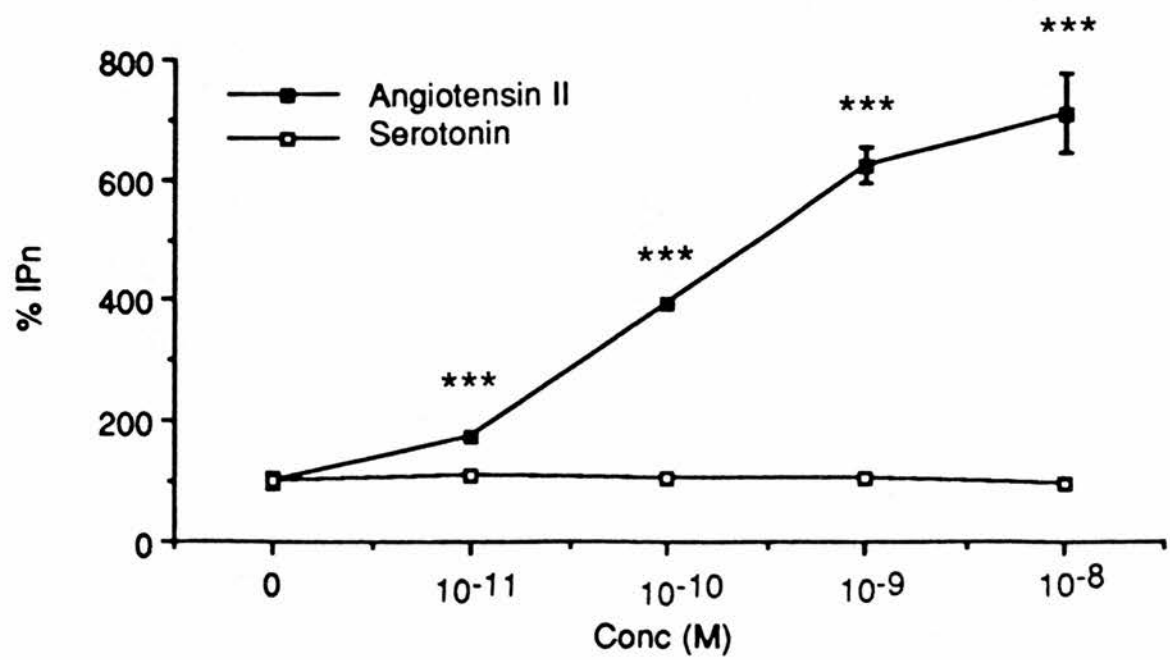


Figure 5.10. **The effect of angiotensin II and serotonin on cellular inositol phosphate content** (n=3)

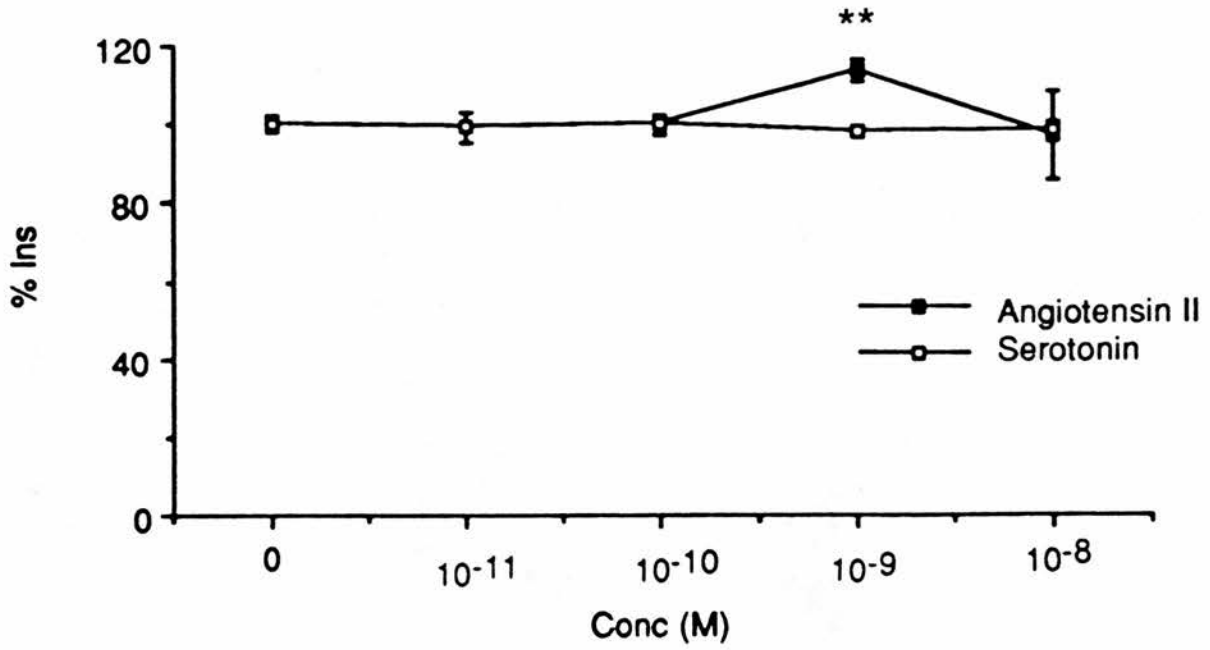


Figure 5.11. **The effect of angiotensin II and serotonin on cellular inositol content** (n=3)

5.6.3. The effect of pertussis toxin on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium.

The participation of a receptor coupled G-protein in mediating the end steroid response was investigated using pertussis toxin, which blocks the inhibitory action of the G_i-protein on adenylate cyclase.

Figure 5.12 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of pertussis toxin (10, 50 ng/ml). The toxin caused a small but significant dose dependent increase in aldosterone secretion stimulated with 10^{-8} M serotonin. However, it failed to show any effect on either basal output or secretion stimulated with 10^{-6} M serotonin.

Figure 5.13 shows a typical aldosterone response to angiotensin II (0, 10^{-10}, 10^{-8} M) in the presence and absence of pertussis toxin (10, 50 ng/ml). The toxin (50 ng/ml) showed a small but significant inhibition of basal aldosterone secretion. Angiotensin II stimulated aldosterone secretion remained unaffected.

Figure 5.14 shows a typical aldosterone response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of pertussis toxin (10, 50 ng/ml). The toxin showed no significant effect on either basal or ACTH stimulated aldosterone secretion.

Figure 5.15 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4, 13.2 mM) in the presence and absence of pertussis toxin (10, 50 ng/ml). The toxin showed no significant effect on either basal or aldosterone secretion stimulated with 5.9 or 8.4 mM potassium. However, a small but significant dose dependent inhibition was observed at 13.2 mM potassium.

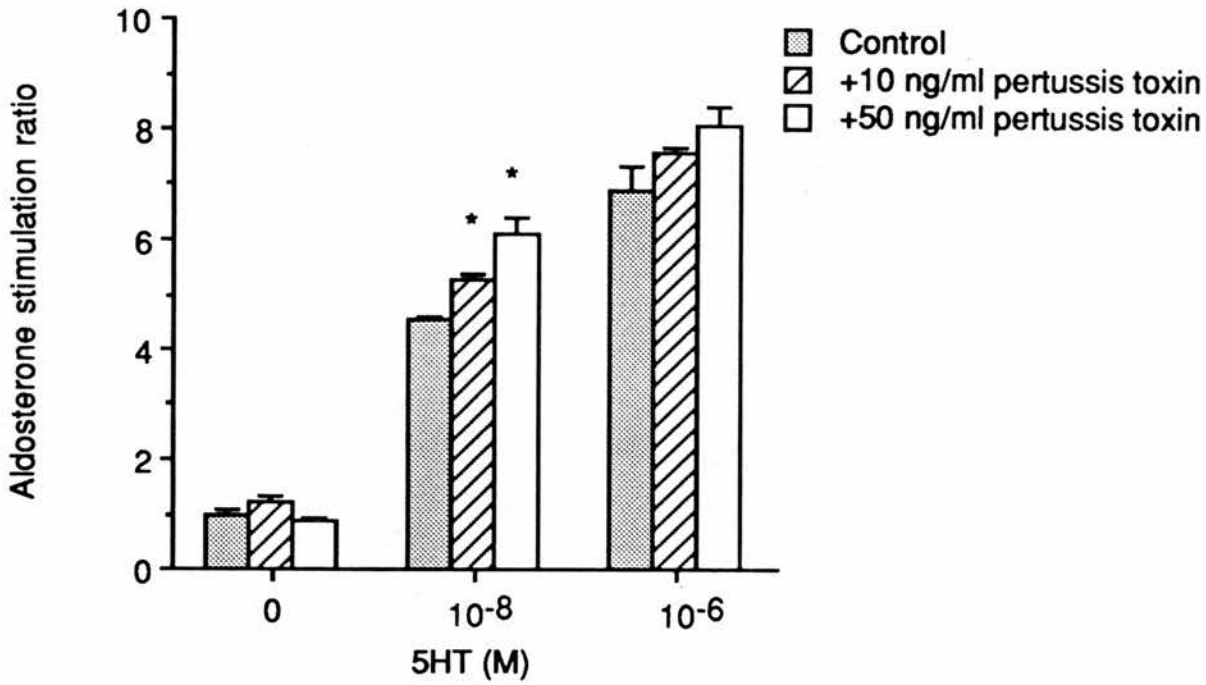


Figure 5.12. **The effect of pertussis toxin on the aldosterone dose response to serotonin.** (n=3)

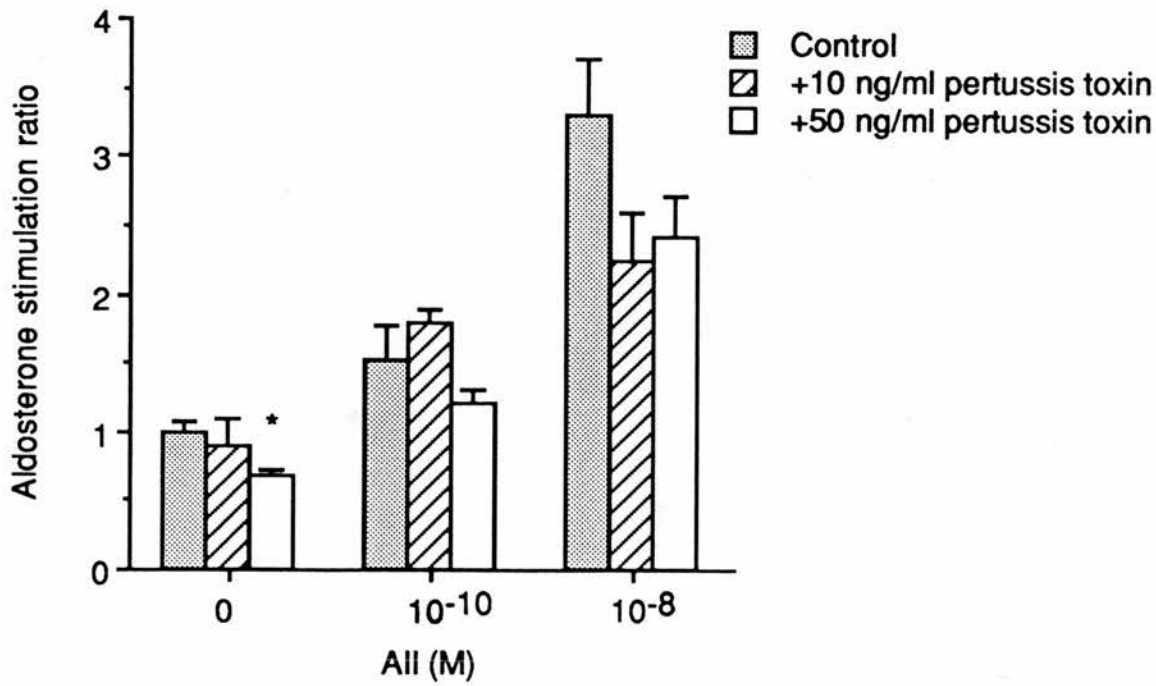


Figure 5.13. **The effect of pertussis toxin on the aldosterone dose response to angiotensin II.** (n=3)

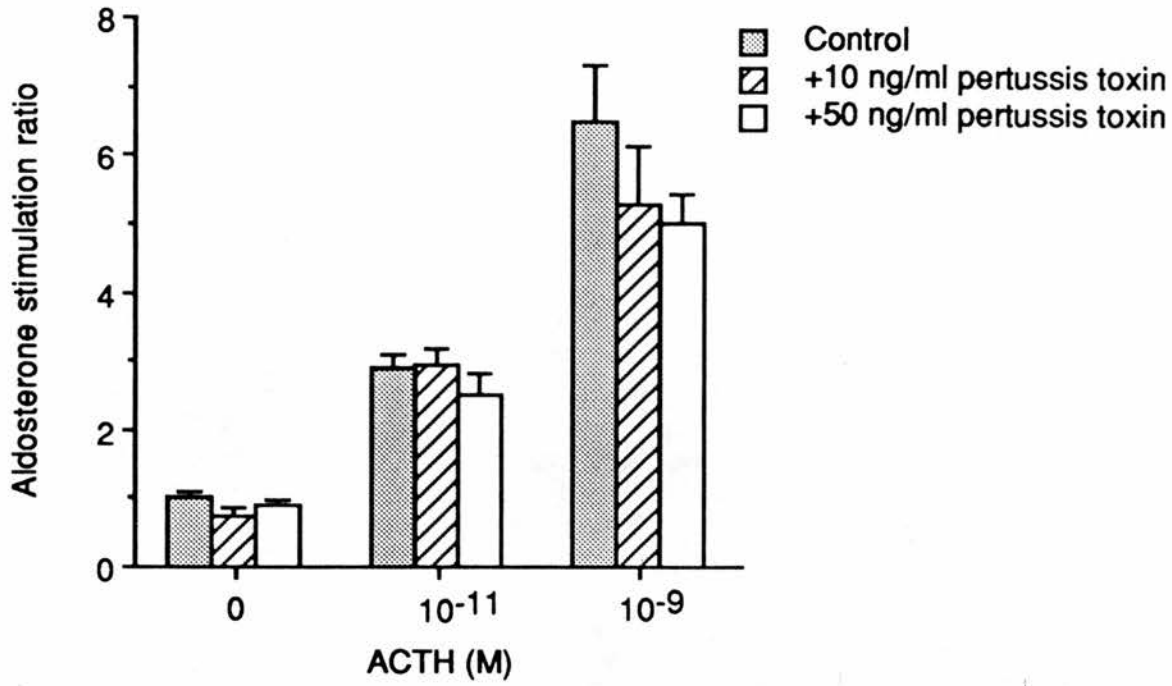


Figure 5.14. **The effect of pertussis toxin on the aldosterone dose response to ACTH.** (n=3)

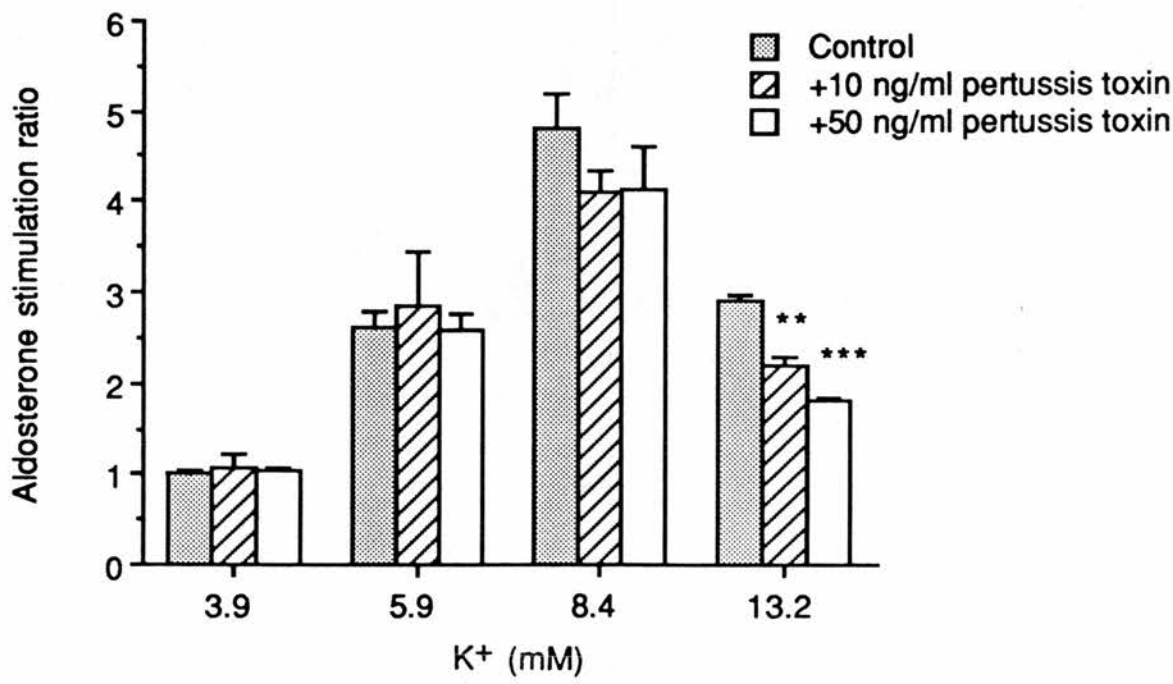


Figure 5.15. **The effect of pertussis toxin on the aldosterone dose response to potassium.** (n=3)

5.6.4. The effect of removal of extracellular calcium on the aldosterone response to serotonin

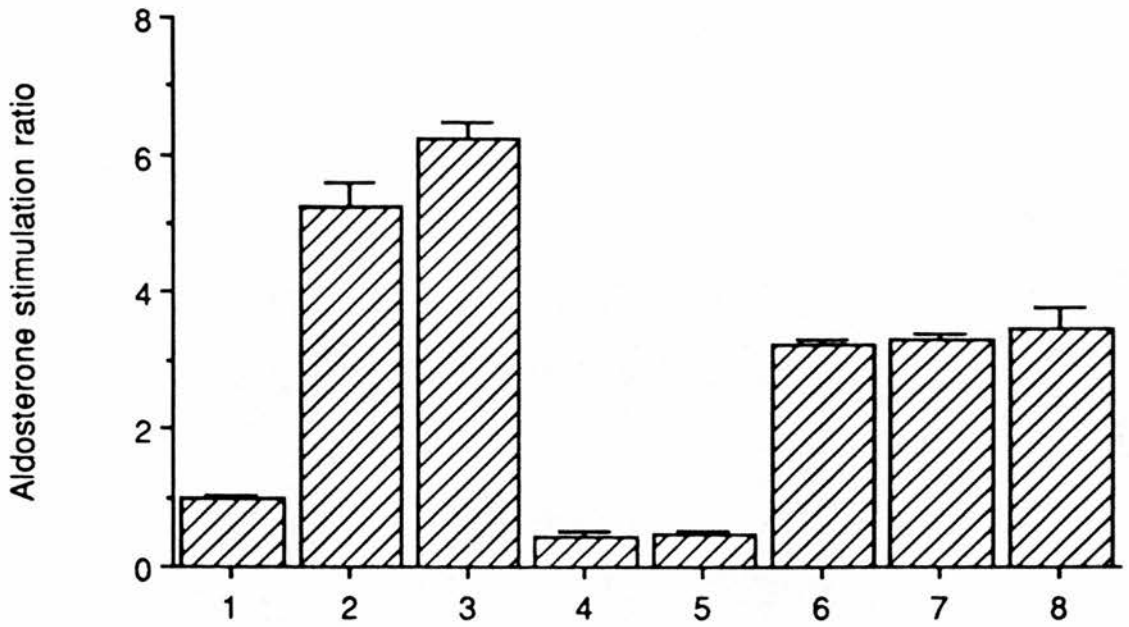
Having established the putative role of cyclic AMP as a second messenger, the role of extracellular Ca^{2+} in the mechanism of action of serotonin was studied by removal of Ca^{2+} from the bathing medium of the cells using the chelating agent EGTA.

Figure 5.16 shows basal (1) and aldosterone secretion stimulated with serotonin (10^{-6} M) (2). Serotonin caused a significant increase in aldosterone secretion ($p<0.001$). The addition of 2.5 mM $CaCl_2$ to the medium had no significant effect on serotoninergic stimulated aldosterone secretion (3). The addition of 5 mM EGTA significantly inhibited aldosterone secretion ($p<0.001$) and this remained inhibited when EGTA was lowered to 2.5 mM ($p<0.001$) (5). Gradual re-introduction of calcium to the medium partially restored the inhibitory effects of EGTA.

5.6.5. The effect of verapamil on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium.

Having determined that extracellular Ca^{2+} is necessary for the steroidogenic action of serotonin, the role of transmembrane Ca^{2+} flux was invesigated using the Ca^{2+} antagonist verapamil, which blocks influx of Ca^{2+} across the plasma membrane via L(long lasting, large capacitance)-type channels.

Figure 5.17 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of verapamil (1.56, 12.5 μM). 12.5 μM verapamil significantly inhibited both basal and serotoninergic stimulated aldosterone secretion. 1.56 μM verapamil also caused significant inhibition of basal and aldosterone stimulated with 10^{-8} M serotonin, but not 10^{-6} M serotonin.



5HT (M)	-	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
Ca^{2+} (mM)	2.5	2.5	5.0	2.5	2.5	3.7	5.0	7.5
EGTA (mM)	-	-	-	5.0	2.5	2.5	2.5	2.5

Figure 5.16. **The effect of removal and gradual re-introduction of extracellular Ca^{2+} on serotoninergic stimulated aldosterone secretion.** (n=3)

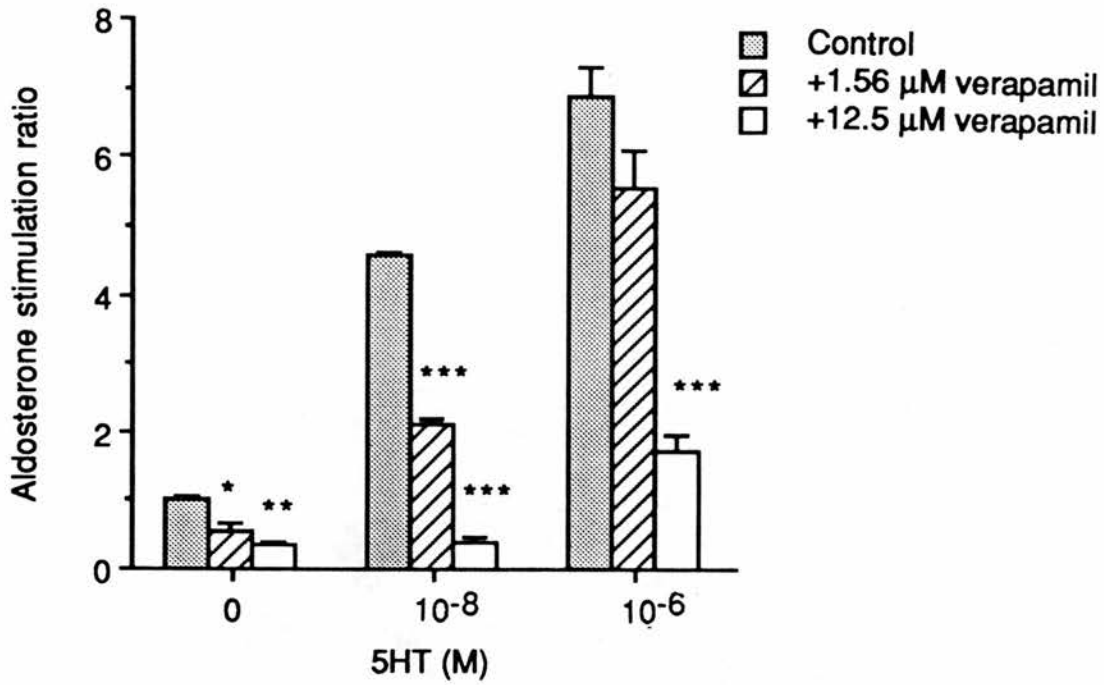


Figure 5.17. **The effect of verapamil on the aldosterone dose response to serotonin.** (n=3)

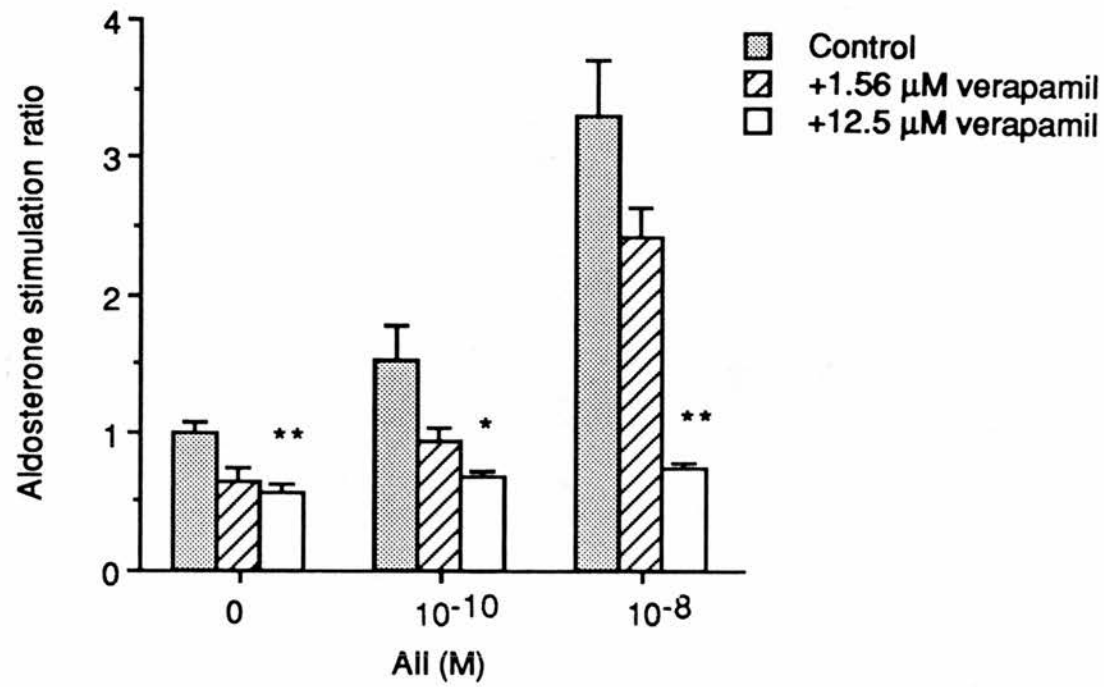


Figure 5.18. **The effect of verapamil on the aldosterone dose response to angiotensin II.** (n=3)

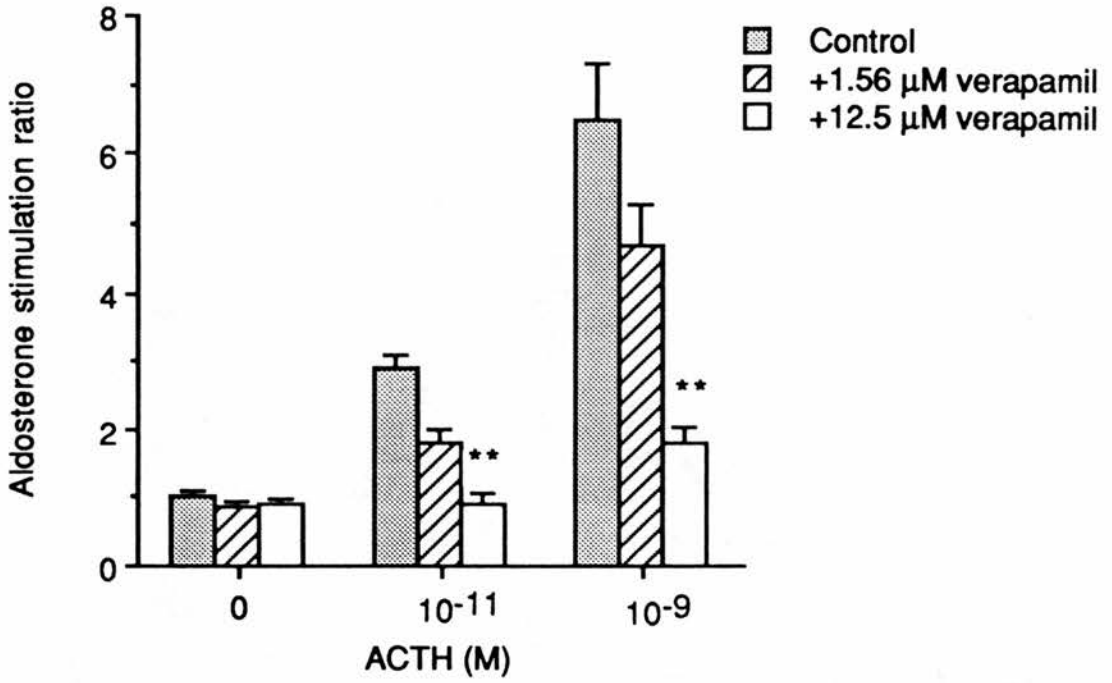


Figure 5.19. **The effect of verapamil on the aldosterone dose response to ACTH.** (n=3)

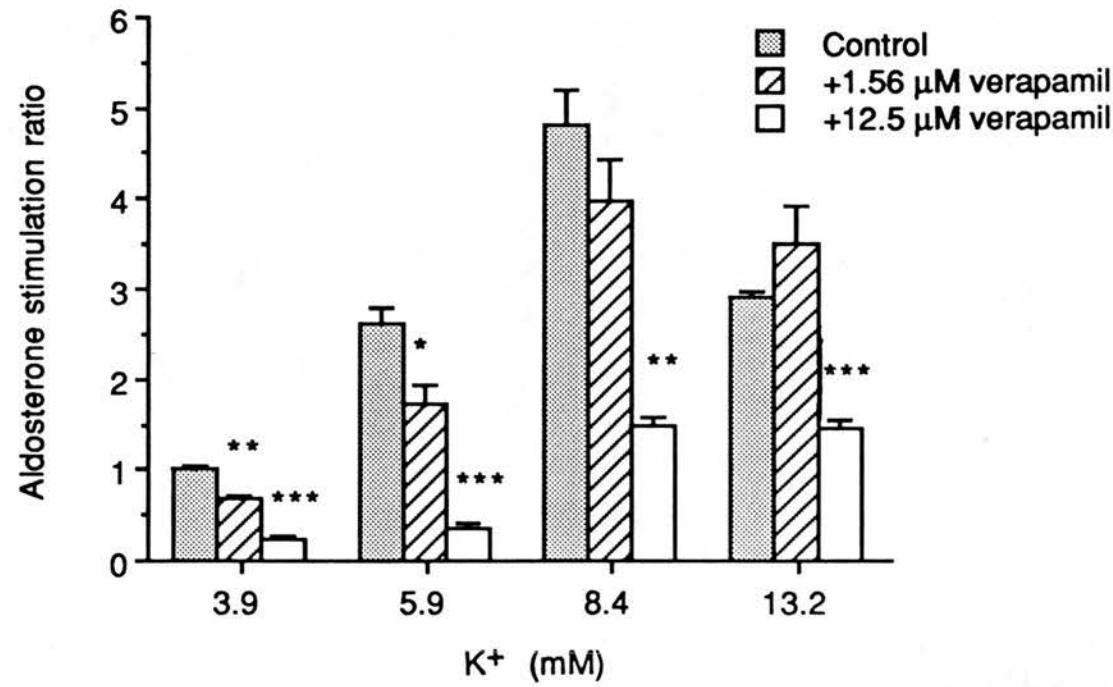


Figure 5.20. **The effect of verapamil on the aldosterone dose response to potassium.** (n=3)

Figure 5.18 shows a typical aldosterone response to angiotensin II (0, 10^{-10}, 10^{-8} M) in the presence and absence of verapamil (1.56, 12.5 μM). 12.5 μM verapamil significantly inhibited both basal and angiotensin II stimulated aldosterone secretion. 1.56 μM verapamil showed no significant effect.

Figure 5.19 shows a typical aldosterone response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of verapamil (1.56, 12.5 μM). 12.5 μM verapamil significantly inhibited ACTH stimulated aldosterone secretion, but had no effect on basal output. 1.56 μM verapamil showed no significant effect.

Figure 5.20 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4, 13.2 mM) in the presence and absence of verapamil (1.56, 12.5 μM). 12.5 μM verapamil significantly inhibited both basal and aldosterone secretion stimulated with potassium. 1.56 μM verapamil significantly inhibited basal and 5.9 mM potassium stimulated secretion, but had no effect on 8.4 or 13.2 mM potassium stimulated output.

5.6.6. The effect of nifedipine on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium.

A similar series of experiments was conducted using another Ca^{2+} antagonist nifedipine, which also acts on the L-type channel, but is considered to be more selective.

Figure 5.21 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of nifedipine (12.5, 50 μM). Nifedipine caused a marked dose dependent significant inhibition of both basal and serotoninergic stimulated aldosterone secretion.

Figure 5.22 shows a typical aldosterone response to angiotensin II (0, 10^{-10}, 10^{-8} M) in

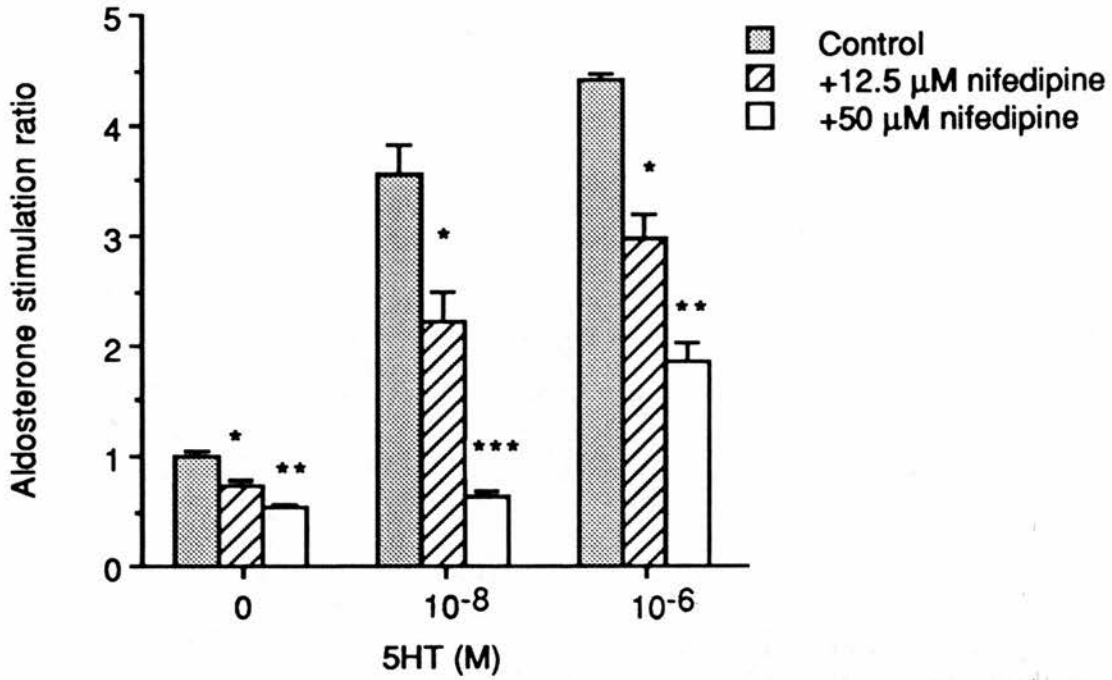


Figure 5.21. **The effect of nifedipine on the aldosterone dose response to serotonin.**
(n=3)

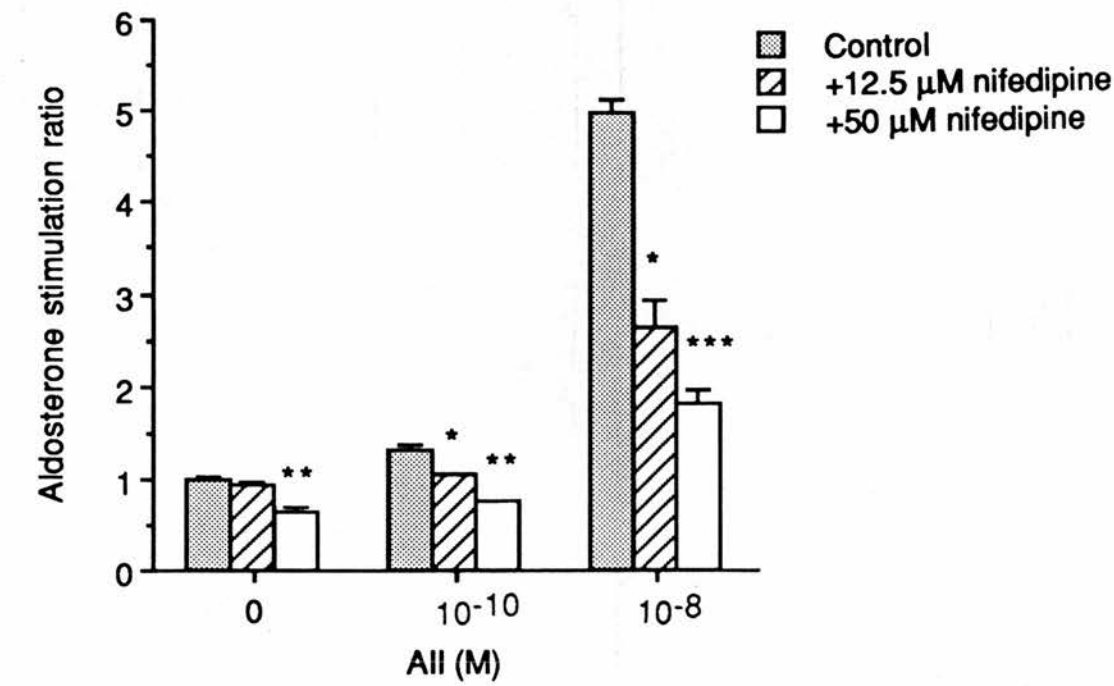


Figure 5.22. **The effect of nifedipine on the aldosterone dose response to angiotensin II.**
(n=3)

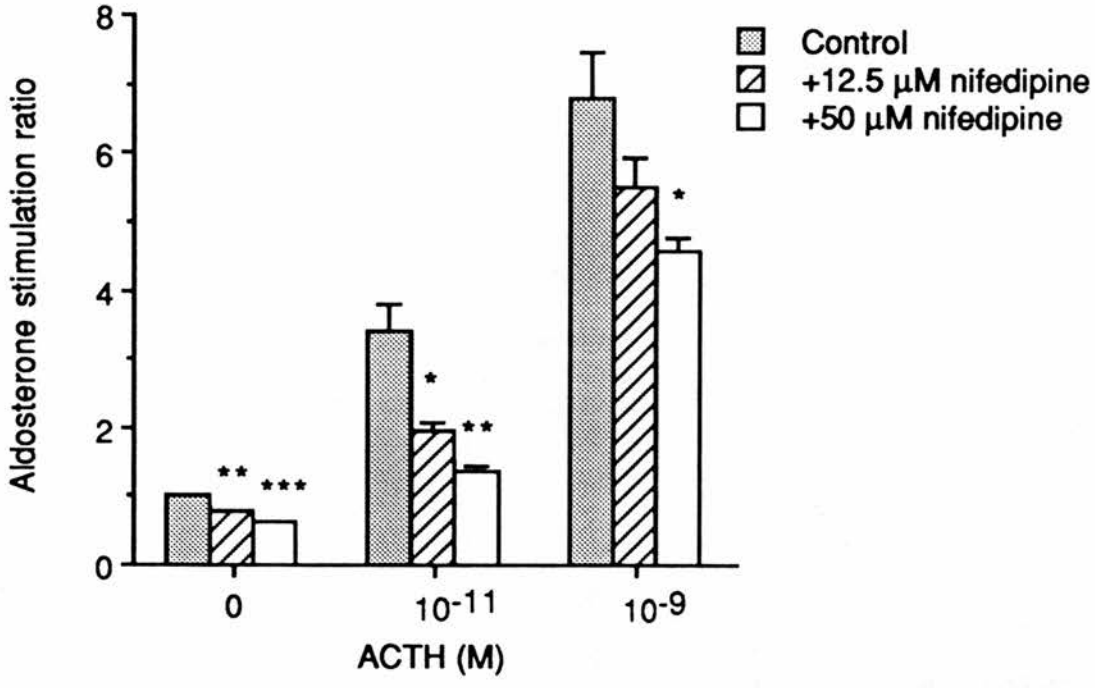


Figure 5.23. **The effect of nifedipine on the aldosterone dose response to ACTH.** (n=3)

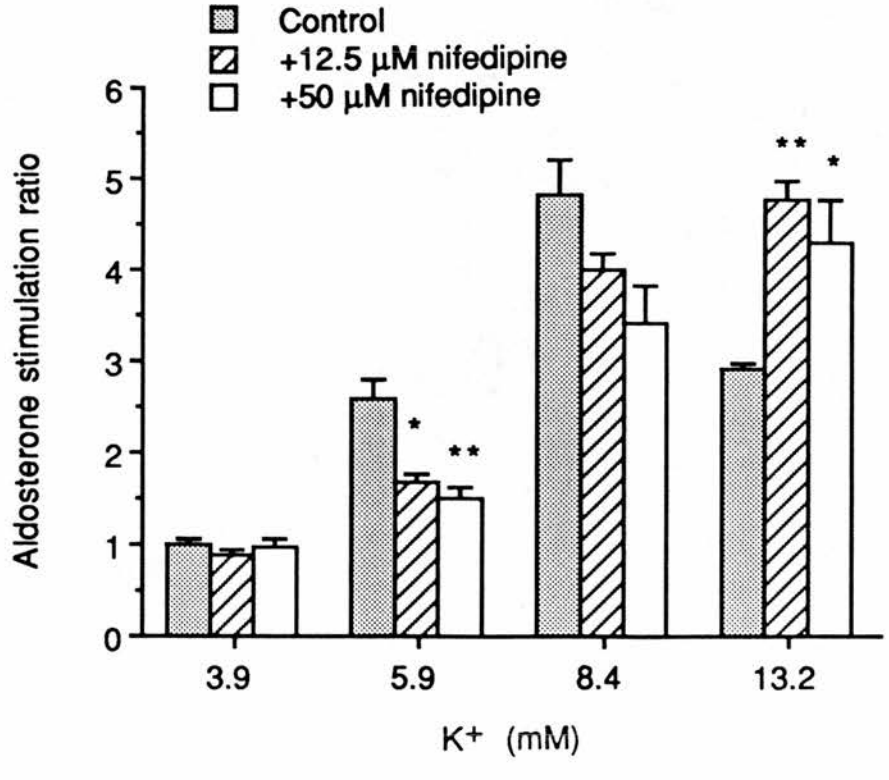


Figure 5.24. **The effect of nifedipine on the aldosterone dose response to potassium.** (n=3)

the presence and absence of nifedipine (12.5, 50 μM). Nifedipine caused a marked dose dependent significant inhibition of both basal and angiotensin II stimulated aldosterone secretion.

Figure 5.23 shows a typical aldosterone response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of nifedipine (12.5, 50 μM). Nifedipine caused a marked dose dependent significant inhibition of basal and aldosterone secretion stimulated with 10^{-11} M ACTH. 50μM nifedipine sustained the inhibition at 10^{-9} M ACTH. However, 12.5 μM nifedipine was ineffective at this concentration of agonist.

Figure 5.24 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4, 13.2 mM) in the presence and absence of nifedipine (12.5, 50 μM). Nifedipine showed no significant effect on basal aldosterone secretion. A dose dependent inhibition of aldosterone secretion by nifedipine was observed at 5.9 mM potassium however, this was not sustained at 8.4 mM potassium. At 13.2 mM potassium, nifedipine at 12.5 and 50 μM showed a marked dose dependent stimulatory effect on aldosterone secretion.

5.6.7. The effect of TMB-8 on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium.

It is clear that extracellular Ca^{2+} plays a key role in the action of serotonin. The release of Ca^{2+} from intracellular storage sites, such as the ER, has been shown to be an important component in the mechanism of action of angiotensin II. The role of intracellular Ca^{2+} in the action of serotonin was studied using TMB-8, which blocks release of calcium from intracellular organelles.

Figure 5.25 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of TMB-8 (1.56, 12.5 μM). TMB-8 showed a marked dose-dependent

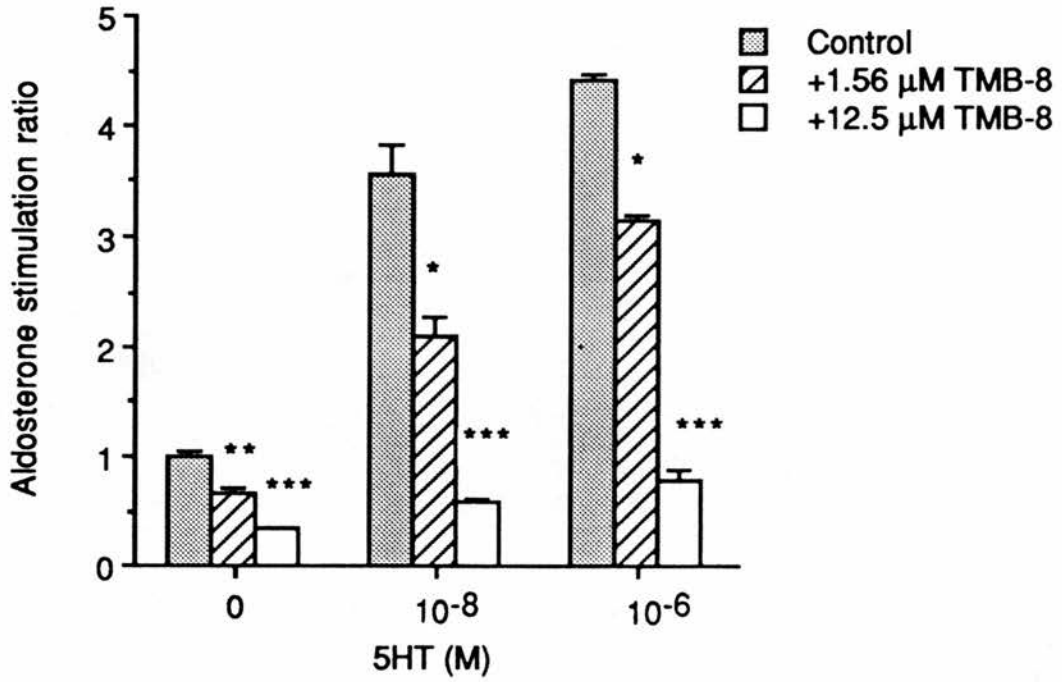


Figure 5.25. **The effect of TMB-8 on the aldosterone dose response to serotonin.** (n=3)

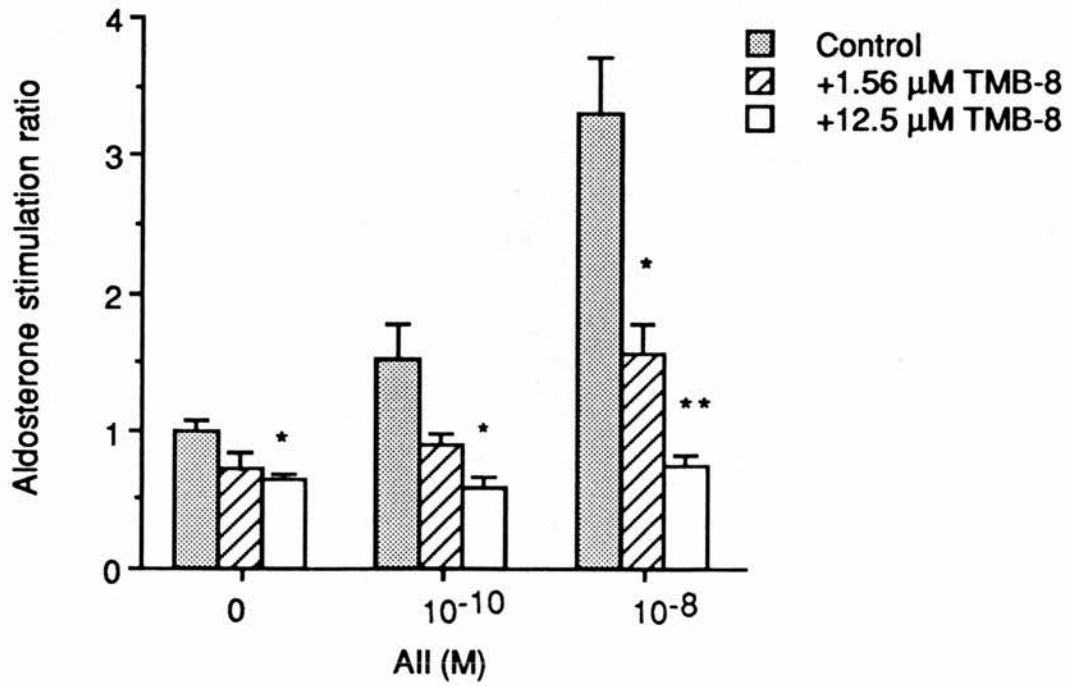


Figure 5.26. **The effect of TMB-8 on the aldosterone dose response to angiotensin II.** (n=3)

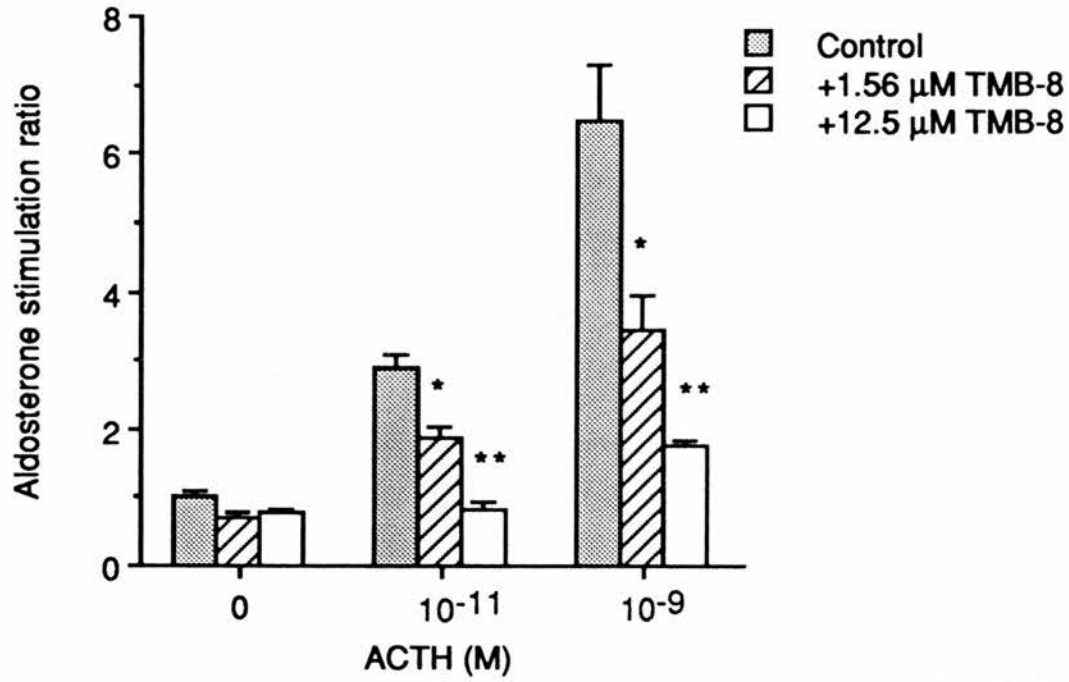


Figure 5.27. **The effect of TMB-8 on the aldosterone dose response to ACTH.** (n=3)

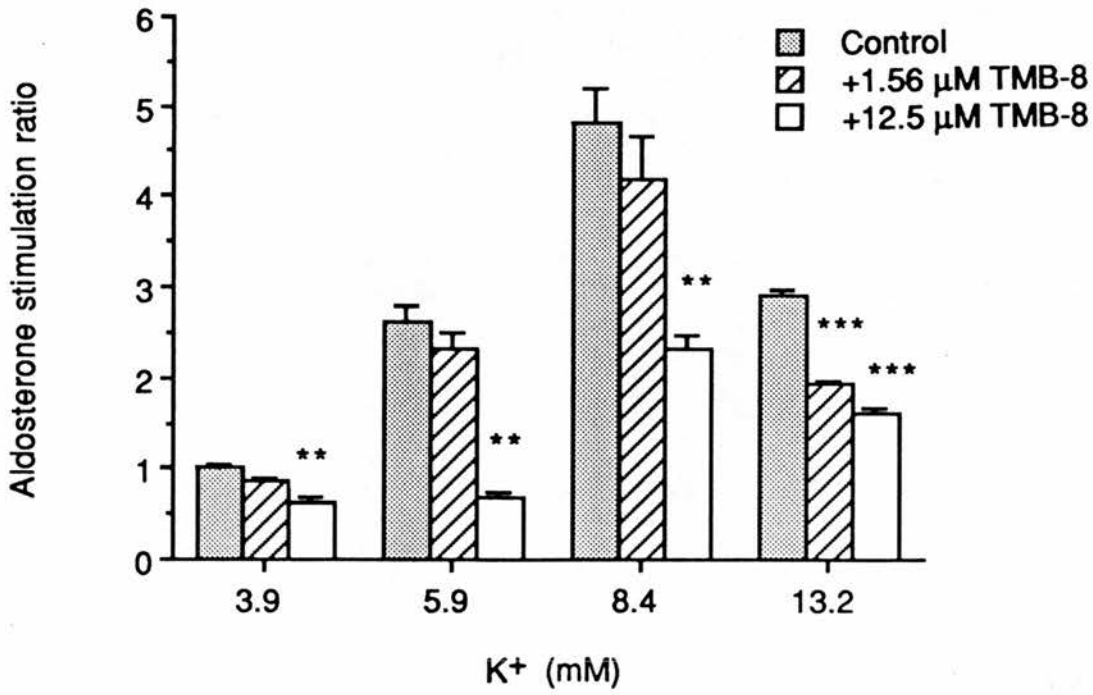


Figure 5.28. **The effect of TMB-8 on the aldosterone dose response to potassium.** (n=3)

inhibition of both basal and serotoninergic stimulated aldosterone secretion.

Figure 5.26 shows a typical aldosterone response to angiotensin II (0, 10^{-10}, 10^{-8} M) in the presence and absence of TMB-8 (1.56, 12.5 μM). TMB-8 showed a marked dose-dependent inhibition of both basal and angiotensin II stimulated aldosterone secretion.

Figure 5.27 shows a typical aldosterone response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of TMB-8 (1.56, 12.5 μM). TMB-8 showed a marked dose-dependent inhibition of ACTH stimulated aldosterone secretion. No significant effect was observed on basal secretion.

Figure 5.28 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4,13.2 mM) in the presence and absence of TMB-8 (1.56, 12.5 μMl). 12.5 μM TMB-8 caused significant inhibition of basal and potassium stimulated aldosterone secretion. 1.56 μM TMB-8 showed no significant inhibition except at 13.2 mM potassium.

5.6.8. The effect of trifluoperazine on the aldosterone dose response to serotonin, angiotensin II, ACTH and potassium.

The liberation of calcium as a second messenger must be accompanied by its binding to a protein, which is rendered active and free to activate the cascade mechanism resulting in steroidogenesis. One such binding protein is calmodulin, whose activity can be blocked by phenothiazines such as trifluoperazine (TFP). The role of calmodulin in the action of serotonin was monitored by studying the effects of TFP on serotoninergic stimulated aldosterone secretion.

Figure 5.29 shows a typical aldosterone response to serotonin (0, 10^{-8}, 10^{-6} M) in the presence and absence of trifluoperazine (1.56, 12.5 μM). 12.5μM TFP showed a marked

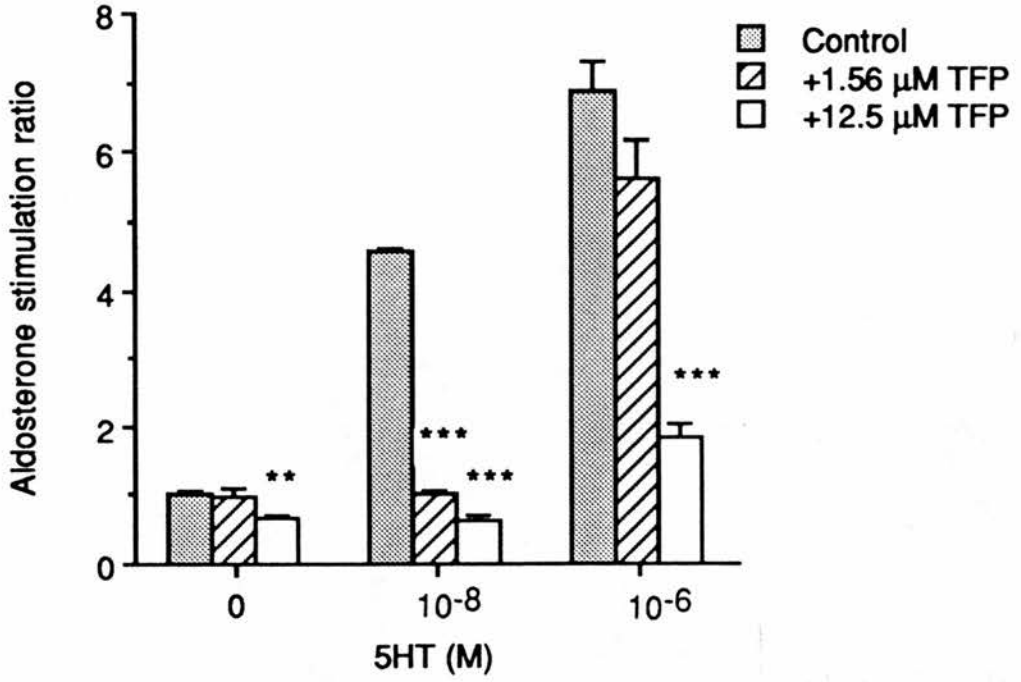


Figure 5.29. **The effect of trifluoperazine on the aldosterone dose response to serotonin.** (n=3)

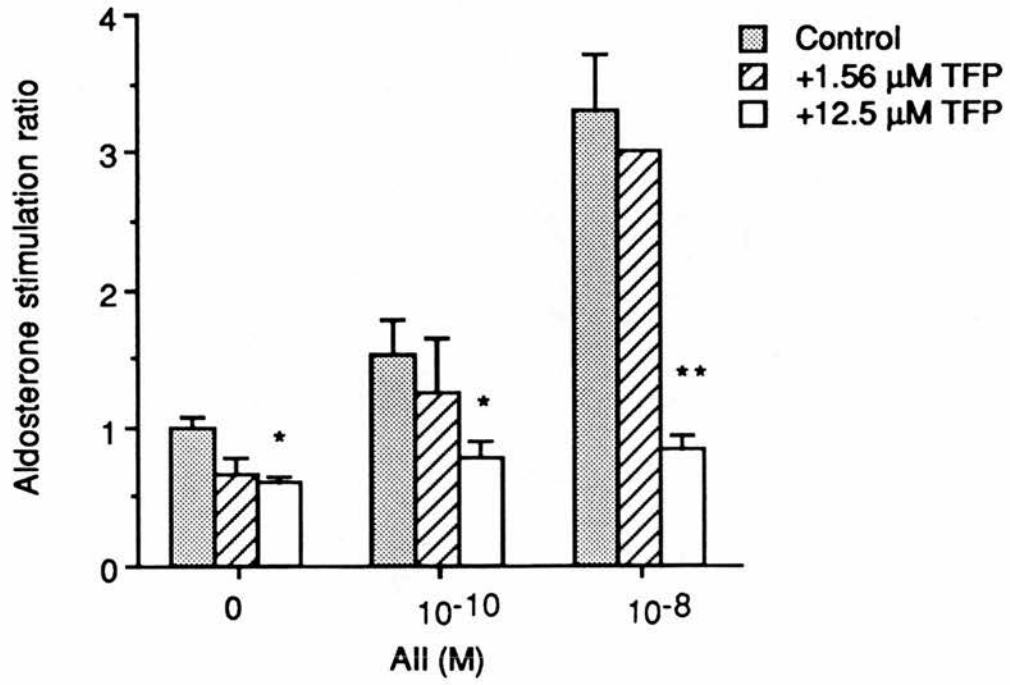


Figure 5.30. **The effect of trifluoperazine on the aldosterone dose response to angiotensin II.** (n=3)

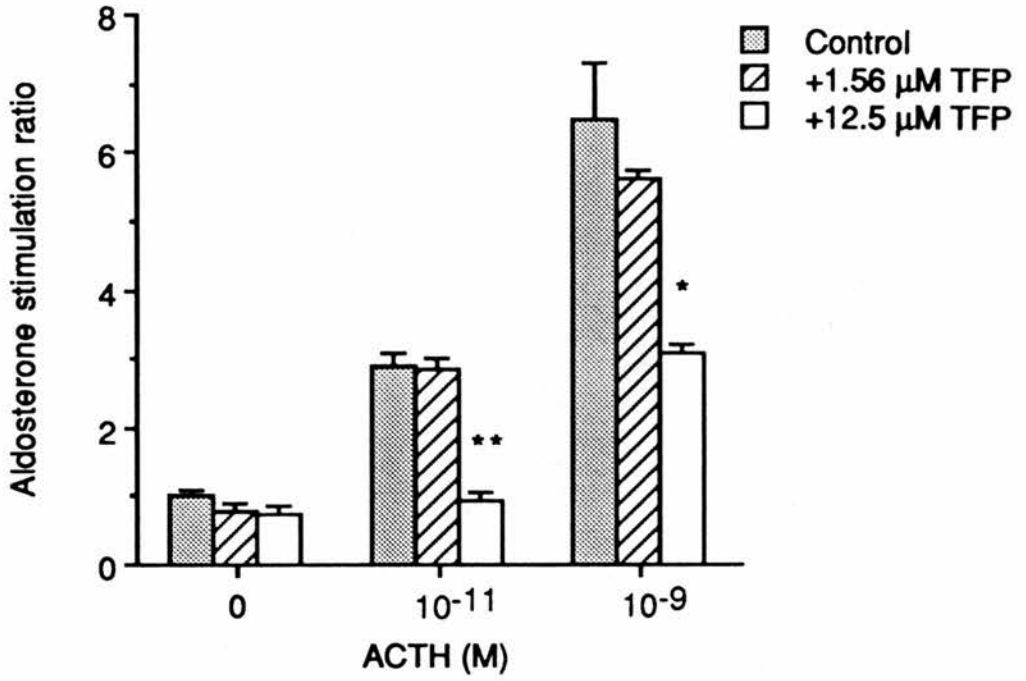


Figure 5.31. **The effect of trifluoperazine on the aldosterone dose response to ACTH.**
(n=3)

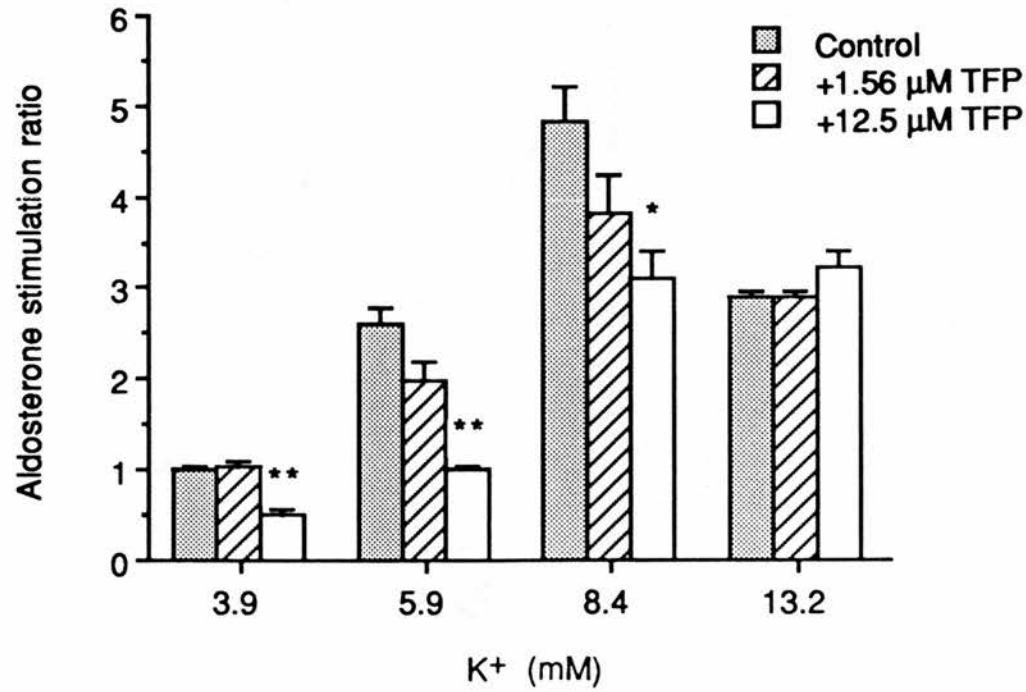


Figure 5.32. **The effect of trifluoperazine on the aldosterone dose response to potassium.**
(n=3)

significant inhibition of both basal and serotoninergic stimulated aldosterone production. 1.56 μM TFP significantly inhibited aldosterone production stimulated with 10^{-8} M serotonin, but had no effect on 10^{-6} M serotonin or basal secretion.

Figure 5.30 shows a typical aldosterone response to angiotensin II (0, 10^{-10}, 10^{-8} M) in the presence and absence of TFP (1.56, 12.5 μM). 12.5 μM TFP showed significant inhibition of both basal and aldosterone secretion stimulated with angiotensin II. 1.56 μM showed no significant inhibition.

Figure 5.31 shows a typical aldosterone response to ACTH (0, 10^{-11}, 10^{-9} M) in the presence and absence of TFP (1.56, 12.5 μM). 12.5 μM TFP showed a significant inhibitory effect on the aldosterone response to ACTH, though no effect on basal secretion. 1.56 μM TFP showed no significant inhibition.

Figure 5.32 shows a typical aldosterone response to potassium (3.9, 5.9, 8.4,13.2 mM) in the presence and absence of TFP (1.56, 12.5 μM). 12.5 μM TFP significantly inhibited basal (3.9 mM) and aldosterone secretion stimulated by 5.9 and 8.4 mM potassium. 1.56 μM TFP showed no significant inhibition.

5.6.9. Radiolabelled calcium uptake studies

Although the previous experiments indicate that calcium plays an important role as a second messenger in the action of serotonin, they are largely indirect pharmacological studies and therefore open to the non-specific and possible toxic actions of the drugs utilised. In order to investigate the role of calcium more directly, radiolabelled calcium influx studies were carried out in isolated cells using $^{45}CaCl_2$.

Figure 5.33 shows the uptake of $^{45}Ca^{2+}$ over a 105 second time course by

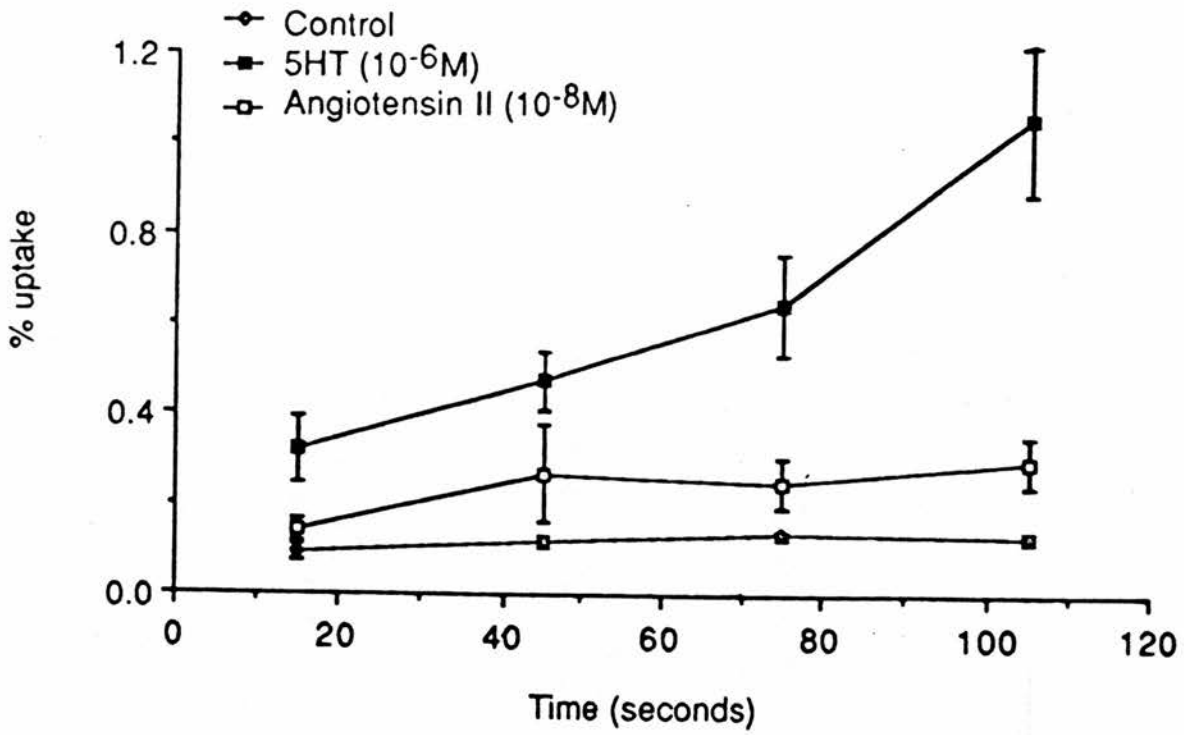


Figure 5.33.

The effect of 5HT and angiotensin II on $^{45}Ca^{2+}$ uptake.

(n=5)

unstimulated cells, those stimulated with serotonin (10^{-6} M) and angiotensin II (10^{-8} M) from 5 combined experiments. The uptake was calculated as a % of the total number of radioactive counts added to each incubation. Compared to the control, serotonin caused a significant uptake of $^{45}Ca^{2+}$ within 15 seconds of addition ($p<0.05$). This uptake continued at 45, 75 and 105 seconds ($p<0.001$), ($p<0.01$) and ($p<0.001$) respectively. Compared to the control, angiotensin II also caused a significant increase in $^{45}Ca^{2+}$ uptake. However, the effect was much slower and was not statistically significant until 105 seconds. Comparison of the uptake between serotonin and angiotensin II revealed that at 15, 75 and 105 seconds, serotonin caused a significantly greater uptake of $^{45}Ca^{2+}$ ($p<0.05$),($p<0.01$) and ($p<0.01$) respectively.

Figure 5.34 shows the uptake of $^{45}Ca^{2+}$ over a 105 second time course by unstimulated cells, those stimulated with serotonin (10^{-6} M) and those stimulated with serotonin in the presence of the Ca^{2+} antagonist verapamil (12.5 μM) from 3 combined experiments. As before, compared to the control, serotonin caused a significant uptake of $^{45}Ca^{2+}$ within 15 seconds ($p<0.05$) and this continued at 45, 75 and 105 seconds ($p<0.05$), ($p<0.001$) and ($p<0.01$) respectively. The addition of verapamil caused significant inhibition of serotoninergic stimulated $^{45}Ca^{2+}$ uptake at 75 and 105 seconds ($p<0.001$) and ($p<0.01$) respectively. The effect of verapamil at 15 and 45 seconds was marginally outside the significance cut off of 0.05. There was no significant difference between the control curve and the serotonin plus verapamil curve.

Figure 5.35 shows the uptake of $^{45}Ca^{2+}$ over a 105 second time course by unstimulated cells, those stimulated with serotonin (10^{-6} M) and those stimulated with serotonin in the presence of the serotonin antagonist mesulergine (10^{-6} M) from 2 combined experiments. As before serotonin caused a significant increase in $^{45}Ca^{2+}$ uptake within 15 seconds ($p<0.05$) and this continued at 45, 75 and 105 seconds ($p<0.05$), ($p<0.01$) and ($p<0.05$) respectively. The addition of mesulergine showed a tendency to inhibit

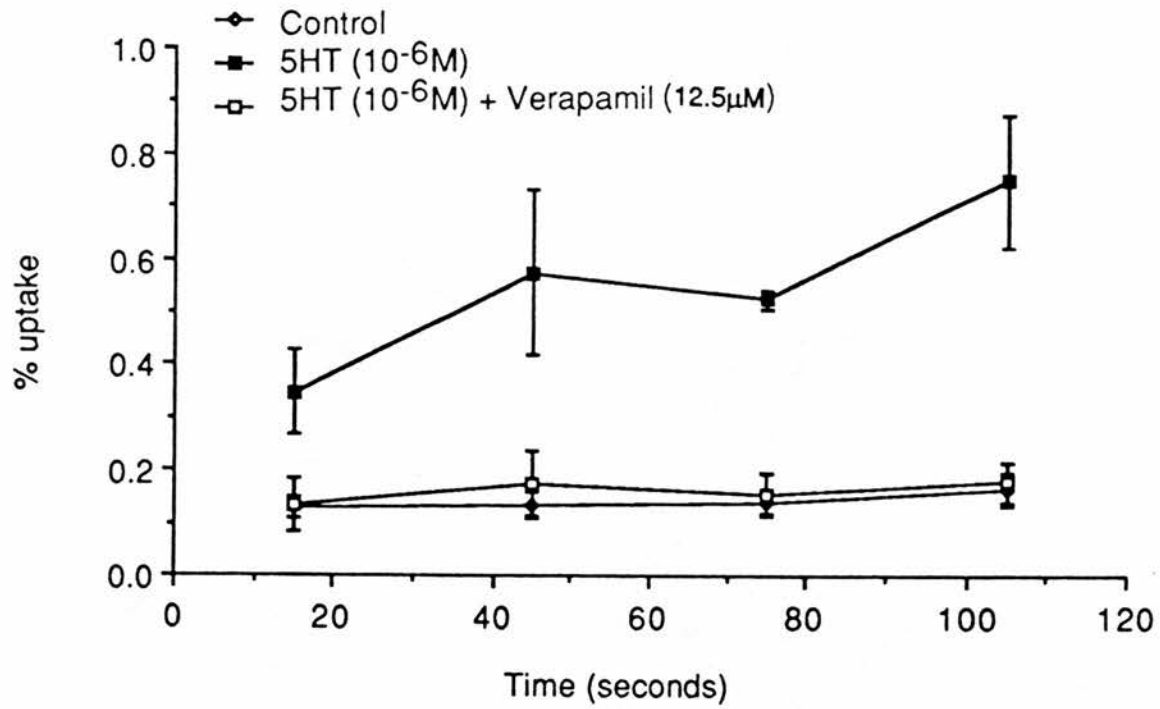


Figure 5.34.

The effect of verapamil on serotoninergic stimulated $^{45}Ca^{2+}$ uptake.
(n=3)

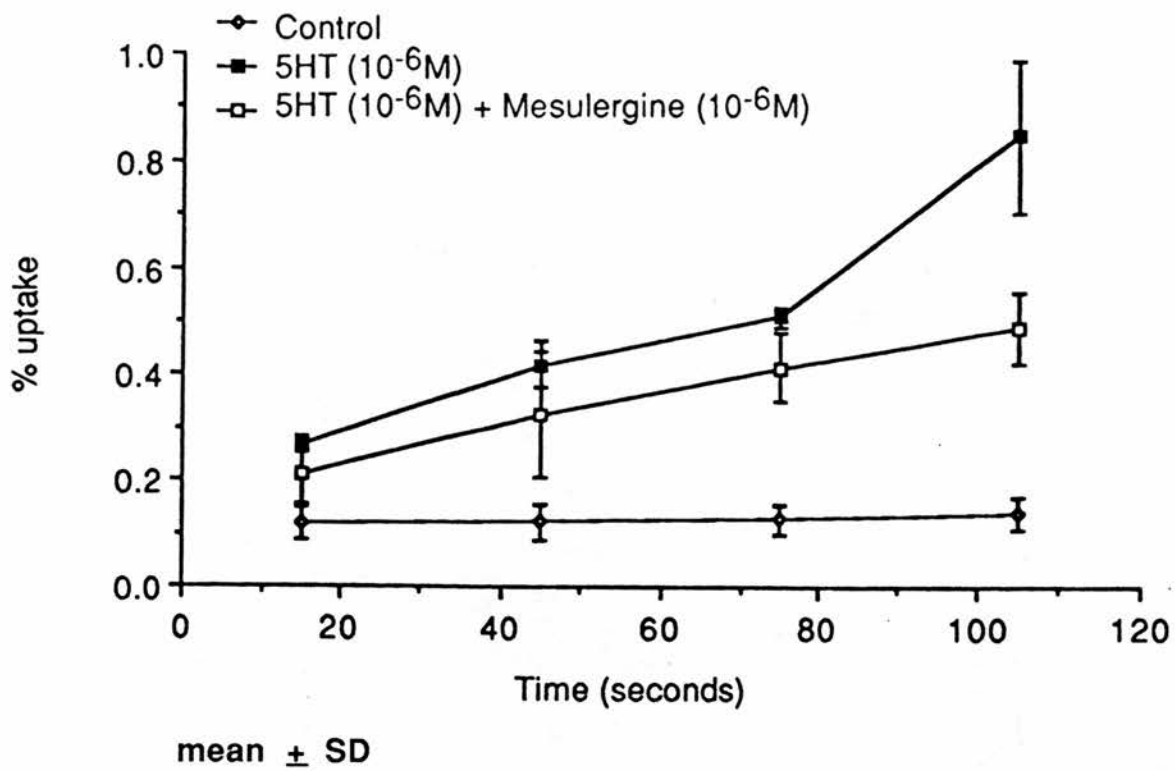


Figure 5.35.

The effect of mesulergine on serotoninergic stimulated $^{45}Ca^{2+}$ uptake. (n=2)

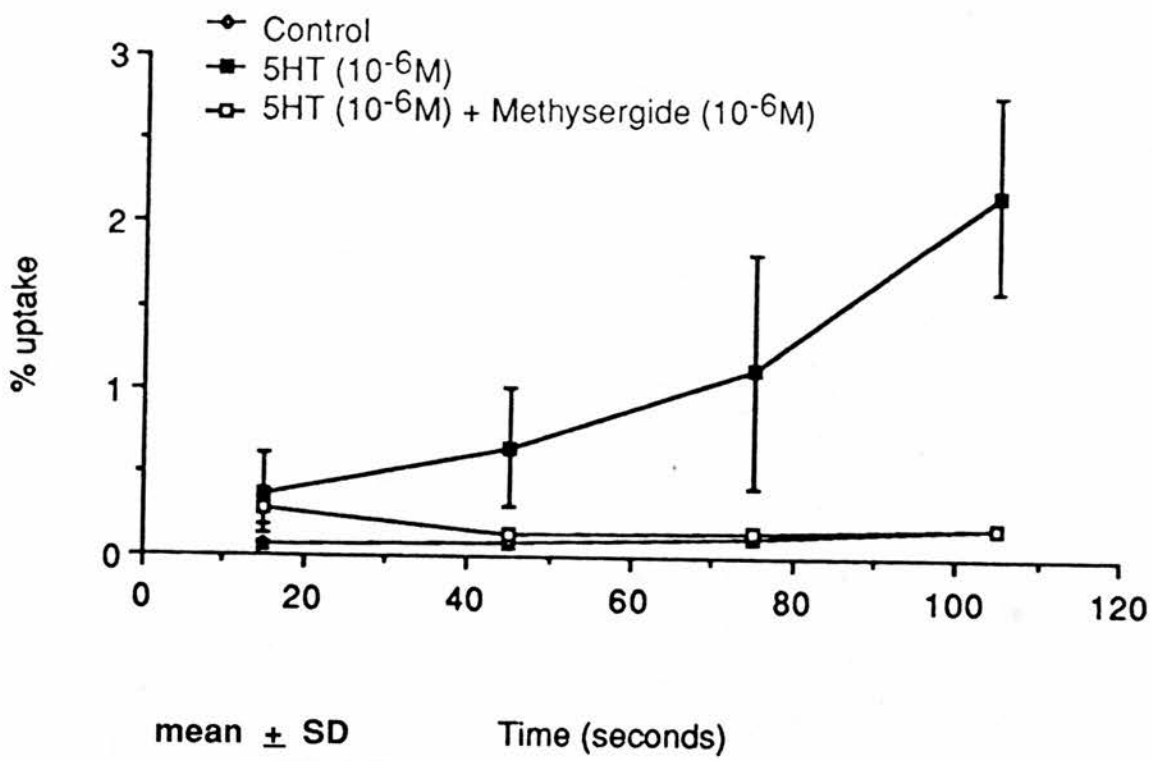
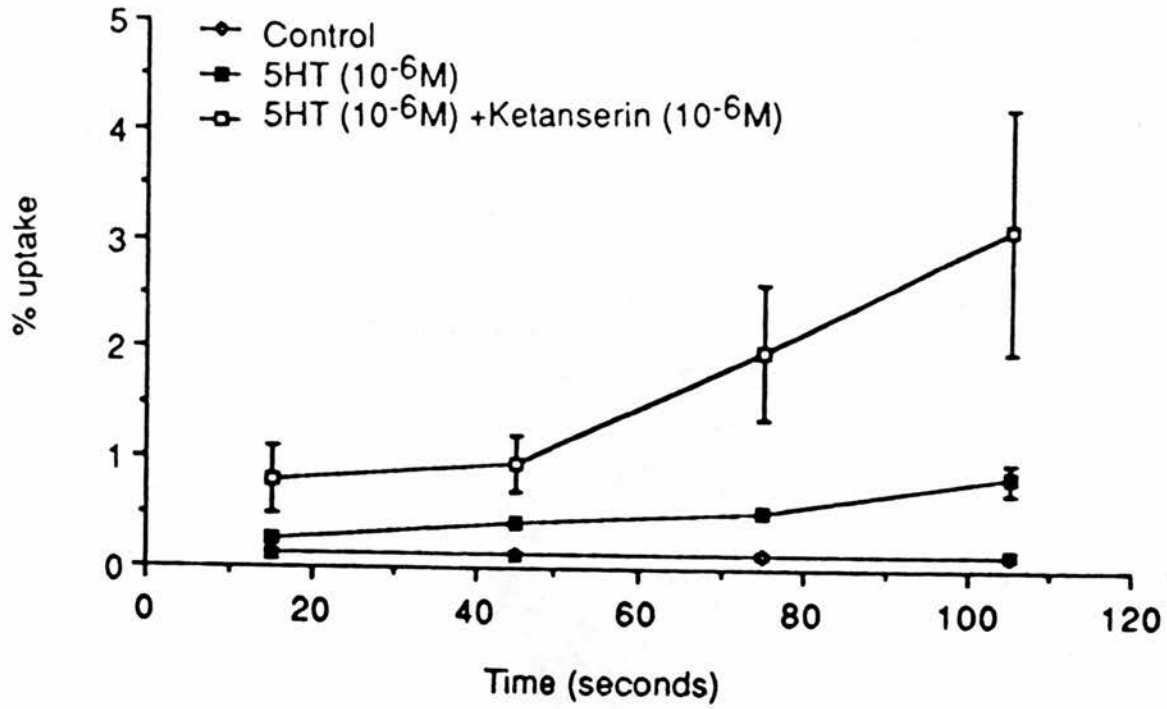


Figure 5.36.

The effect of methysergide on serotoninergic stimulated $^{45}Ca^{2+}$ uptake. (n=2)



(mean ± SD)

Figure 5.37.

The effect of ketanserin on serotoninergic stimulated $^{45}Ca^{2+}$ uptake. (n=2)

serotoninergic stimulated $^{45}Ca^{2+}$ uptake. However, the effect was not significant, probably due to the large error bars and small experimental number.

Figure 5.36 shows the uptake of $^{45}Ca^{2+}$ over a 105 second time course by unstimulated cells, those stimulated with serotonin (10^{-6} M) and those stimulated with serotonin in the presence of the serotonin antagonist methysergide (10^{-6} M) from 2 combined experiments. As before serotonin caused a significant increase in $^{45}Ca^{2+}$ uptake within 15 seconds ($p<0.05$) and this continued at 45, 75 and 105 seconds ($p<0.05$), ($p<0.01$) and ($p<0.05$) respectively. The addition of methysergide showed a tendency to inhibit serotoninergic stimulated $^{45}Ca^{2+}$ uptake. However, the effect was not significant until 105 seconds ($p<0.05$).

Figure 5.37 shows the uptake of $^{45}Ca^{2+}$ over a 105 second time course by unstimulated cells, those stimulated with serotonin (10^{-6} M) and those stimulated with serotonin in the presence of the serotonin antagonist ketanserin (10^{-6} M) from 2 combined experiments. As before serotonin caused a significant increase in $^{45}Ca^{2+}$ uptake within 15 seconds ($p<0.05$) and this continued at 45, 75 and 105 seconds ($p<0.05$), ($p<0.01$) and ($p<0.05$) respectively. In contrast, the addition of ketanserin showed a tendency to increase $^{45}Ca^{2+}$ uptake. However, the effect was not significant, probably due to the large error bars and small experimental number.

5.7. Discussion

Having established the possible existence of specific receptors for serotonin within the adrenal zona glomerulosa, by basic pharmacological studies, more definitive proof of the presence of receptors requires the existence of a second messenger system which acts as a coupling mechanism between initial hormone / receptor interaction and end steroid response, thus enabling the transformation of an initially small signal to a large end response

by a complex cascade mechanism.

The second messenger systems coupled to ACTH and angiotensin II and potassium, the major physiological regulators of aldosterone secretion, have already been studied in great detail by a number of groups and have been thoroughly reviewed in chapter one. It is now generally accepted that ACTH and angiotensin II act through the adenyl cyclase and PI second messenger systems respectively, and each stimulus causes complex ionic changes within the cell. Potassium, in contrast acts predominantly by cellular depolarisation resulting in calcium influx. An increase in cyclic AMP is also observed, but it is unclear if this is the primary event or secondary to calcium influx. Similarly, the second messenger systems coupled to serotonin receptors within the CNS and the cardiovascular system have also been well characterised. The $5HT_2$ and $5HT_{1c}$ receptors are coupled to the PI system, whilst the other receptors are known to modulate adenyl cyclase activity (Enjalbert *et al* 1978, Peroutka *et al* 1981, Conn and Sanders Bush 1984, De Chaffoy de Courcelles 1985). This chapter has attempted to elucidate the second messenger system coupled to serotonin receptors in the zona glomerulosa and compare these with the results obtained for angiotensin II, ACTH and potassium and serotonin within other systems.

The results clearly illustrate that serotonin stimulates cyclic AMP production, indicating that in the adrenal, the serotonin receptor, which is most probably $5HT_{1c}$ / $5HT_2$ like, is positively coupled to the adenylate cyclase second messenger system. This is in direct contrast to $5HT_2$ and $5HT_{1c}$ receptors within the CNS and the cardiovascular system, which act through the PI system (De Chaffoy de Courcelles 1985), but is consistent with the results obtained by a number of other groups (Albano *et al* 1974, Fujita *et al* 1979, Matsuoka *et al* 1985). The stimulation ratio is however much less than that observed with ACTH. It may be argued that the increase in cyclic AMP following serotoninergic stimulation results from the stimulation of contaminating zona fasciculata cells. However, this is unlikely as the stimulatory action of serotonin is unique to the zona glomerulosa (Müller *et al* 1970, Haning *et al* 1970).

A further experiment using dideoxyadenosine, which supposedly inhibits adenyl cyclase, was carried out to ascertain the role of cyclic AMP in the action of serotonin. However, at the concentration used (10^{-6} M), the drug had no effect on steroidogenesis stimulated with either serotonin or ACTH which acted as a control.

It is of interest that the concentration of serotonin required to stimulate cyclic AMP is much higher than that needed to increase steroidogenesis, indicating a clear dissociation between second messenger and end response. A similar effect was observed for ACTH in adrenocortical cells obtained from decapsulated adrenal gland by other groups who demonstrated a discrepancy between adenylate cyclase activity and steroidogenesis. They also showed that specific binding of ACTH to cells correlates well with cyclic AMP production, but not steroidogenesis (Beall and Sayers 1972, Mackie *et al* 1972, Buckley and Ramachandran 1981). These results strongly support the receptor reserve model by Hornsby and Gill in 1978, which suggested that only a small fraction of cyclic AMP formed in response to hormonal stimulation is required for steroidogenesis.

In addition to cyclic AMP, measurement of various components of the PI system were also carried out. Serotonin had no effect on lipid content, or PI turnover within the cell, indicating that serotonin acts, like ACTH, exclusively on the adenylate cyclase second messenger system. In contrast, angiotensin II, which acted as an experimental control as it is known to act through the PI system, had no effect on cyclic AMP production, but did significantly decrease the % lipid content of the cell, in parallel to increasing the concentration of IP_3.

A secondary series of experiments using pertussis toxin and quinacrine (data not shown) were also designed to completely eliminate the PI system in the action of serotonin. Pertussis toxin ADP-ribosylates the α-subunit of the G_i-protein, therefore blocking its inhibitory action on adenylate cyclase, whilst quinacrine supposedly inhibits phospholipase activity. Therefore, if a stimulus utilises the G_i protein, the toxin should inhibit

steroidogenesis. Pertussis toxin has proved a valuable experimental tool in evaluating the role of the G_i protein in other systems, however, in the adrenal its effects are more difficult to interpret. Pretreatment of zona glomerulosa cells with the toxin causes inhibition of both the angiotensin II induced increase in cytoplasmic calcium and adenylate cyclase activity, without having any effect on IP_3 production or aldosterone secretion (Kojima *et al* 1986). This has led to the suggestion that an unidentified G -protein mediates the action of angiotensin II and that inhibition of adenylate cyclase is not a critical component of the steroid response (Ross and Gilman 1980, Woodcock and Johnston 1984, Kojima *et al* 1986, Hausdorff *et al* 1987). Similarly, the results obtained in this study were difficult to interpret and it was impossible to derive any meaningful conclusions from the data as neither pertussis toxin nor quinacrine showed any consistent significant effect on the aldosterone dose response to serotonin or angiotensin II. It may be relevant that the design of the experiments was incorrect, as other studies pre-incubate cells with the toxin for periods of up to 1 hour, whereas in these studies the cells were not pre-incubated.

Having therefore established that serotonin acts through cyclic AMP, the role of calcium as a second messenger was also studied. The experiment using the chelating agent EGTA, which removes calcium from the extracellular medium, suggests that serotonin requires the presence of extracellular calcium to stimulate aldosterone secretion. Gradual re-introduction of calcium to the bathing medium is sufficient to return the steroidogenic response to almost normal. A similar effect of EGTA on serotonin induced aldosterone secretion was shown by Ganguly and Hampton in 1985. In experiments carried out by other groups EGTA abolished the sustained phase of hormone secretion in response to angiotensin II, whereas the initial rapid phase was unaffected (Spät 1988). However, it is as yet unclear whether the response to serotonin is bi-phasic like angiotensin II or monophasic and sustained like ACTH (Kojima *et al* 1985a). Unfortunately cyclic AMP was not measured in this particular study, as it would have provided a clear indication of whether extracellular calcium influx was required at a site

proximal or distal to cyclic AMP formation.

Ca^{2+} is therefore necessary in the incubation media for the action of serotonin. However, it is unclear if it acts in a permissive and / or mediatory capacity. The permissive role of calcium has been shown in the action of ACTH by radioligand binding studies in zona fasciculata cells using an iodinated analogue in the presence of EGTA. These studies demonstrated that both receptor binding and steroidogenesis exhibit an absolute requirement for extracellular calcium, and addition of calcium restored both functions in parallel (Cheitlin *et al* 1985). Until formal binding studies have been carried out using a suitable serotonin radioligand the requirement of Ca^{2+} for the binding of serotonin to its receptor cannot be assessed. The non-permissive role of Ca^{2+} in the action of serotonin was investigated by indirect means using Ca^{2+} channel antagonists and directly by measurement of $^{45}Ca^{2+}$ influx.

Verapamil and nifedipine are calcium antagonists which act on the L (long lasting, large capacitance)-type calcium channels as opposed to the T (transient) and N (neuronal) channels, although nifedipine is generally considered to be more selective. At the higher concentration verapamil caused significant inhibition of the dose response to all the stimuli. At the lower dose, verapamil was only an effective inhibitor at the lower dose of serotonin, and had no effect on the response to angiotensin II and ACTH, although the response to potassium was inhibited except at 13.2 mM. Nifedipine was used at higher concentrations, and caused significant inhibition of the aldosterone response to serotonin and angiotensin II. The response to ACTH was inhibited by the higher concentration of nifedipine, however the smaller dose only inhibited at the lower dose of ACTH. The response to potassium was only inhibited at 5.9 mM by both concentrations of nifedipine, and enhanced at 13.2 mM.

The results indicate that calcium influx across the cell membrane is a necessary component of the signal transduction mechanism for serotonin. The effect of verapamil on the aldosterone response to serotonin, angiotensin II, ACTH and potassium has also been demonstrated by other groups (Balla *et al* 1982a, Ganguly and Hampton 1985). The inhibitory

capacity of nifedipine was less than that of verapamil, consequently higher concentrations of nifedipine had to be utilised. This difference in inhibitory properties of each antagonist may reflect different modes of action, it is already known that verapamil acts on open channels, whereas the dihydropyridine diltiazem, acts on closed channels (Janis and Scriabine 1983). Therefore, it is possible that nifedipine may be more effective in the presence of inactivated channels. It has also been reported that there are a number of sub-types of L-channel, which can be divided on the basis of their opening and closing times, conductance and selectivity to various toxins. In addition, it is possible that verapamil exhibits a number of other cellular effects, such as the inhibition of calmodulin (Janis and Scriabine 1983). The limited inhibitory effect of nifedipine on the steroid response to potassium may indicate that Ca^{2+} influx in this system is primarily via another calcium channel. The enhancing effect of nifedipine observed at 13.2 mM potassium, where the control steroid stimulatory response is in fact attenuated, is not easy to explain and is only observed at the high potassium concentration when the zona glomerulosa cell membrane is hyper-depolarised.

Once inside the cell, it is presumed that Ca^{2+} must interact with a binding protein in order to mediate its action. One such binding protein is calmodulin, which has no intrinsic enzyme activity, but on binding calcium it undergoes a conformational change that alters its association with other components of the signalling system. In order to investigate the role of calmodulin in the action of serotonin, trifluoperazine (TFP), an inhibitor of calmodulin, which inhibits the binding of Ca^{2+}, was incubated with the cells, and the results were compared with those of angiotensin II, ACTH and potassium. High concentrations of TFP inhibited the dose response to serotonin and the other stimuli. However, the lower dose of TFP only inhibited steroidogenesis at low concentations of serotonin, and showed no effect on the response to the other secretagogues. A number of other studies using TFP have confirmed that it inhibited the stimulation of aldosterone production by angiotensin II, potassium, prostaglandin E_2 and ouabain, but the effects on ACTH and dibutyryl cyclic AMP stimulated secretion were much less marked (Schifrin *et al* 1982, Balla *et al* 1982a, 1982b).

The data suggest that in addition to Ca^{2+} influx across the plasma membrane, activation of calmodulin is a necessary component in the mechanism of action of serotonin. It can be assumed that the Ca^{2+} from the extracellular source may bind to and activate calmodulin. However, it is also possible that there is release of Ca^{2+} from an intracellular storage site, such as the ER, following hormone / receptor interaction, as is the case with angiotensin II. This hypothesis was tested using TMB-8, which inhibits intracellular Ca^{2+} release. Both concentrations of TMB-8 significantly inhibited the aldosterone response to serotonin, angiotensin II and ACTH. Only the higher dose was an effective inhibitor of potassium stimulated steroidogenesis. Although the data may suggest that the liberation of Ca^{2+} from an intracellular source is required for the action of serotonin, the specificity of the compound must be questioned, as the response to ACTH is also inhibited. ACTH is not generally believed to cause release of Ca^{2+} from intracellular storage sites, therefore no inhibition should be observed. Regarding the lack of specificity of the compound, it has recently been reported that TMB-8 possesses both Ca^{2+} and Na^{+} channel blocking properties and also reduces membrane conductance for potassium ions (Himmel and Ravens 1990) and it may be these properties which contrbute to the results obtained in this study. However, the alternative explanation is that cyclic AMP dependent stimuli such as serotonin and ACTH can cause release of calcium from another intracellular pool which is not IP_3 sensitive. Certainly, studies in zona glomerulosa cells with ACTH and the calcium sensitive bioluminescent dye aequorin have shown changes in intracellular free Ca^{2+} concentration (Kojima and Ogata 1986).

Although the pharmacological studies indicate that Ca^{2+} influx is an important part of the action of serotonin, they are indirect studies which are open to the non-specific actions of the drugs utilised. In order to confirm the transmembrane influx of Ca^{2+} in the action of serotonin and compare the influx with that obtained with angiotensin II, radiolabelled Ca^{2+} uptake studies were carried out using $^{45}CaCl_2$, which can be detected inside the cell by

β-counting. The results show that the introduction of serotonin to zona glomerulosa cells caused an immediate uptake of $^{45}Ca^{2+}$, which continued for up to 105 seconds. Angiotensin II also caused $^{45}Ca^{2+}$ uptake, however the effect was of a much smaller magnitude, probably because the Ca^{2+} required for stimulation of steroidogenesis by angiotensin II, is derived predominantly from an intracellular source, although it is clear there is also some, albeit small, extracellular requirement. Angiotensin II has already been shown to cause a sustained increase in Ca^{2+} influx via T-type channels, which can be blocked with saralasin or nitrendipine (Kojima *et al* 1985a, 1987), although in contrast other workers have reported no influx of Ca^{2+}. As expected, the Ca^{2+} influx stimulated by serotonin can be blocked by verapamil and also to some degree by the serotonin antagonists methysergide and mesulergine. In sharp contrast, ketanserin the $5HT_2$ antagonist, promotes a large influx of Ca^{2+} across the cell membrane, even though it inhibits steroidogenesis as chapter 4 illustrates. Ca^{2+} efflux experiments were not carried out as they have already been reported in detail by other groups, who showed that serotonin, ACTH and potassium did not affect Ca^{2+} efflux, unlike angiotensin II (Williams *et al* 1981).

In addition to the second messenger systems studied, it is possible that the action of serotonin is mediated, at least in part, by a process other than those already discussed. Indeed, serotonin has been shown to be rapidly taken up by rat capsular cells, therefore implicating an additional intracellular action of the amine (Trost and Müller 1976). Another possibility is that intracellular potassium may regulate the aldosterone response to serotonin as ouabain which inhibits the (Na,K)-ATPase, inhibits the aldosterone stimulating properties of ACTH and cyclic AMP. However, it has been shown that in the adrenal, both intracellular potassium concentration and the activity of the ATPase was unaffected by serotonin (Mendelsohn and Mackie 1975, Mackie *et al* 1977, Mendelsohn and Warren 1975, Meuli and Müller 1982)

In summary, the adrenal receptors for serotonin, identified on the basis of

pharmacological agents as $5HT_{1c}$ / $5HT_2$ like, seem to be coupled to adenylate cyclase and L-type Ca^{2+} channels which when activated by serotonin allow Ca^{2+} influx. The results suggest that serotonin in the adrenal gland acts, like ACTH, by binding to a specific membrane receptor and activating adenyl cyclase, resulting in an increase in cyclic AMP secretion. Closely coupled to the enzymatic changes are ionic changes, whereby Ca^{2+} moves from the extracellular fluid across the plasma membrane via specific ion channels, where it interacts with the Ca^{2+} binding protein calmodulin, thus initiating the poorly understood cascade mechanism, resulting in steroidogenesis. There is no direct evidence from these studies as such, to suggest that there is also release of Ca^{2+} from intracellular storeage sites such as the ER or mitochondria. Further studies using Ca^{2+} specific fluorescent dyes such as fura-2 may help to elucidate any possible bi-phasic increase in intracellular Ca^{2+}, as has already been carried out extensively for angiotensin II. These studies could be carried out on both cell populations or single cells. It is noteworthy however, that these studies be carried out in a species which is responsive to serotoninergic stimulation, such as the rat, and not the cow, thus avoiding mis-interpretation of the lack of response, as other groups have done (Capponi *et al* 1987).

Chapter Six

Serotoninergic Control of Aldosterone Secretion *In Vivo*.

6.1. Introduction

Although the stimulatory capacity of serotonin on aldosterone secretion *in vitro* is well documented its physiological relevance in the control of mineralocorticoid secretion remains unclear. Several studies have demonstrated that infusions of serotonin or its immediate precursors tryptophan or 5HTP stimulate aldosterone secretion *in vivo* (Modlinger *et al* 1979, Mantero *et al* 1982, Shenker *et al* 1985a, 1985b, Maestri *et al* 1988). However, it is unclear if this is a direct effect of serotonin on the adrenal gland or whether it is mediated indirectly by activation of the renin-angiotensin system, the hypothalamo-pituitary adrenal axis or indeed some other factor such as potassium.

In order to study the effects of acute serotonin enhancement *in vivo*, the effect of 5HTP administration was studied in conscious rats with indwelling arterial cannulae. Similar experiments were then conducted on animals following pharmacological manipulation of the renin-angiotensin system and hypothalamo-pituitary adrenal axis with the ACE inhibitor captopril and the synthetic glucocorticoid dexamethasone respectively.

6.2. Aims of study

1. To study the acute effects of enhanced serotonin status by administration of 5HTP, the immediate precursor of serotonin, on circulating 5HTP, 5HT, 5HIAA, PRA, corticosterone, aldosterone, sodium and potassium concentrations.
2. To study the effect of 5HTP administration on blood pressure.
3. To study the relative contribution of the hypothalamo-pituitary axis in the action of 5HTP by blocking the release of ACTH using the synthetic glucocorticoid dexamethasone.
4. To study the relative contribution of the renin-angiotensin system in the action of 5HTP by pharmacological blockade of the system using the ACE inhibitor captopril.
5. To study the permissive, rather than mediatory, role of angiotensin II in the action of 5HTP, by the co-infusion of angiotensin II together with ACE inhibition.

6.3. Materials and methods

The materials and methods used in this chapter have already been described in detail in chapter 3, sections 3.4 to 3.6. Briefly, serial blood samples were taken from conscious male rats with indwelling arterial cannulae before and after i.p administration of 5HTP or saline. The blood was analysed for 5HTP, serotonin and 5HIAA by HPLC, for PRA, corticosterone and aldosterone by RIA and for sodium and potassium by flame photometry. The experiment was then repeated following the various manipulations of the renin-angiotensin system and hypothalamo-pituitary adrenal axis.

6.4. Statistical analysis

Results are illustrated as mean $\pm$ standard error mean (SEM). Statistical significance between separate groups at the same time point was calculated using Student's t-test for unpaired samples. Significance between different time points in the same group of animals was estimated using Student's t-test for paired samples. A p value of <0.05 was considered significant. NS indicates non-significance. The p values for this study are quoted in the text and are not illustrated in the figures.

6.5. Results

6.5.1. Study 1. The effect of 5HTP or saline administration on untreated animals

This preliminary study illustrates the changes in the various parameters in two groups of animals (n=4/group), following i.p administration of either saline (1 ml 0.9% w/v) or 5HTP (4 mg/ml), given immediately after collection of the basal blood sample.

Figure 6.1 shows the serum 5HTP concentration in both groups of animals throughout the experimental time course. The basal levels of 5HTP were not significantly different in

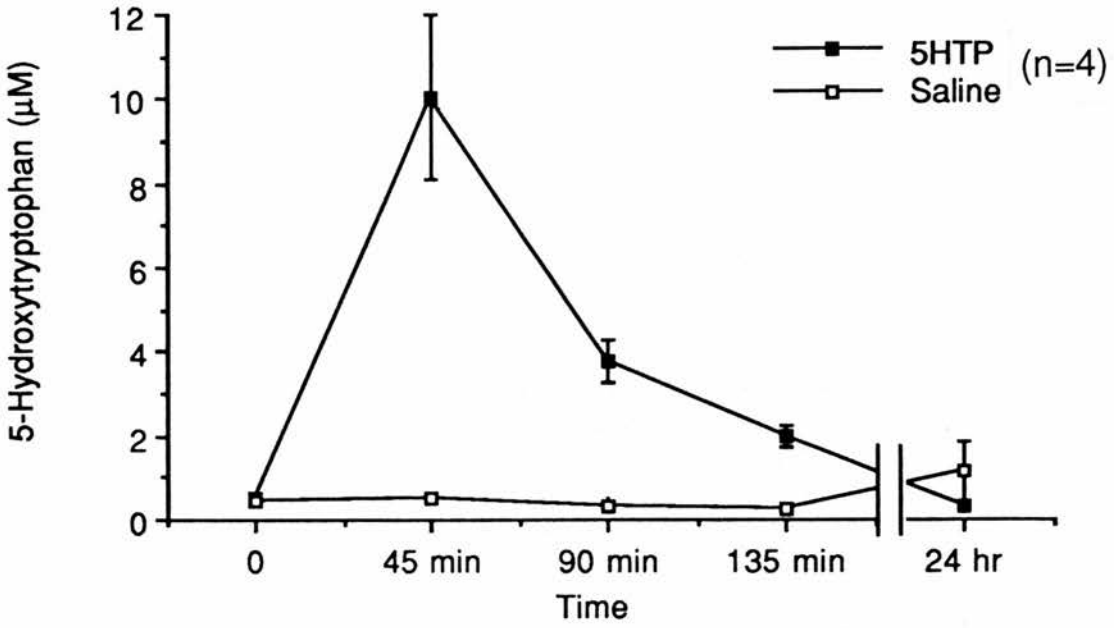


Figure 6.1. **The effect of saline or 5HTP injection on serum 5HTP concentration in the cannulated rat.**

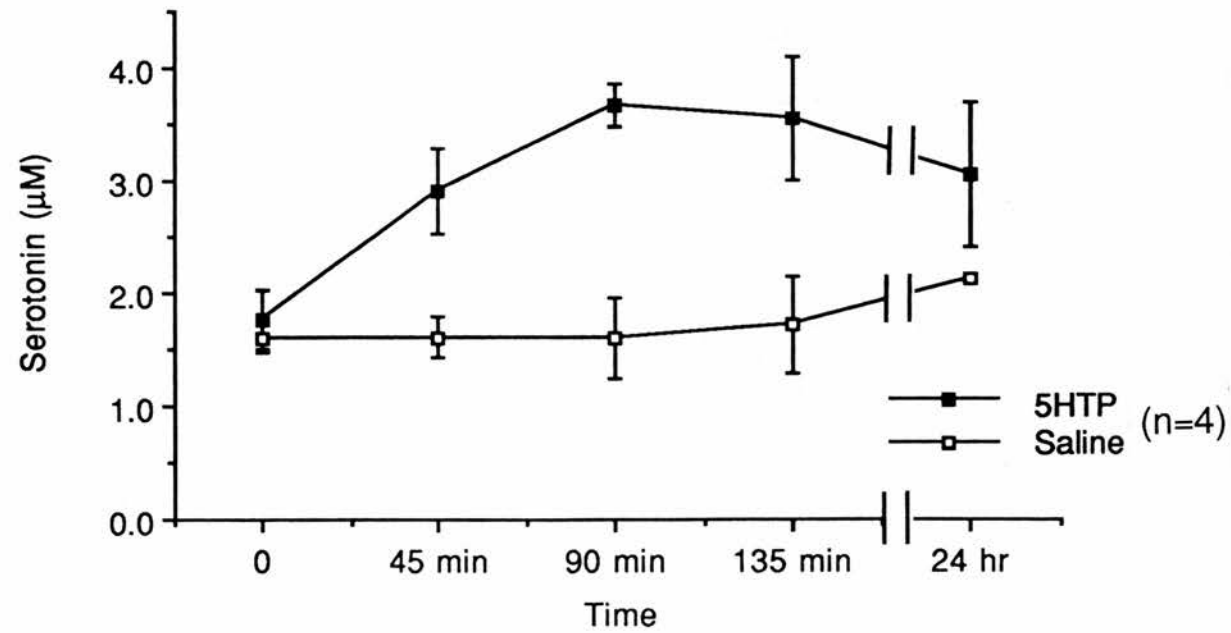


Figure 6.2. **The effect of saline or 5HTP injection on serum serotonin concentration in the cannulated rat.**

each group. Following 5HTP administration, 5HTP levels increased maximally from 0.5 ± 0.1 to 10.0 ± 2.0 μM ($p<0.05$) at 45 minutes and returned to basal values within 24 hours. Administration of saline showed no significant effect on 5HTP levels.

Figure 6.2 shows the serum serotonin concentration in both groups of animals throughout the experimental time course. The basal levels of serotonin were not significantly different in each group. Following 5HTP administration, serotonin levels increased maximally from 1.8 ± 0.3 to 3.7 ± 0.2 μM ($p<0.05$) at 90 minutes and returned to basal values within 24 hours. Administration of saline showed no significant effect on serotonin levels.

Figure 6.3 shows the serum 5HIAA concentration in both groups of animals throughout the experimental time course. The basal levels of 5HIAA were not significantly different in each group. Following 5HTP administration, 5HIAA levels increased maximally from 0.23± 0.1 to 9.2 ± 2.2 μM ($p<0.05$) at 45 minutes and returned to basal values within 24 hours. Administration of saline showed no significant effect on 5HIAA levels.

Figure 6.4 shows the PRA in both groups of animals throughout the experimental time course. Basal PRA was not significantly different in each group. Following 5HTP administration, PRA increased maximally from 1.4 ± 0.5 to 29.8 ± 9.5 ng/ml/hr ($p<0.05$) at 45 minutes and returned to basal values within 135 minutes. Administration of saline showed no significant effect on PRA.

Figure 6.5 shows the plasma corticosterone concentration in both groups of animals throughout the experimental time course. The basal levels of corticosterone were not significantly different in each group. Following 5HTP administration, corticosterone increased maximally from 60.0 ± 23.6 to 729.2 ± 217.0 nM ($p<0.05$) at 45 minutes and returned to basal values within 135 minutes. Administration of saline showed no significant

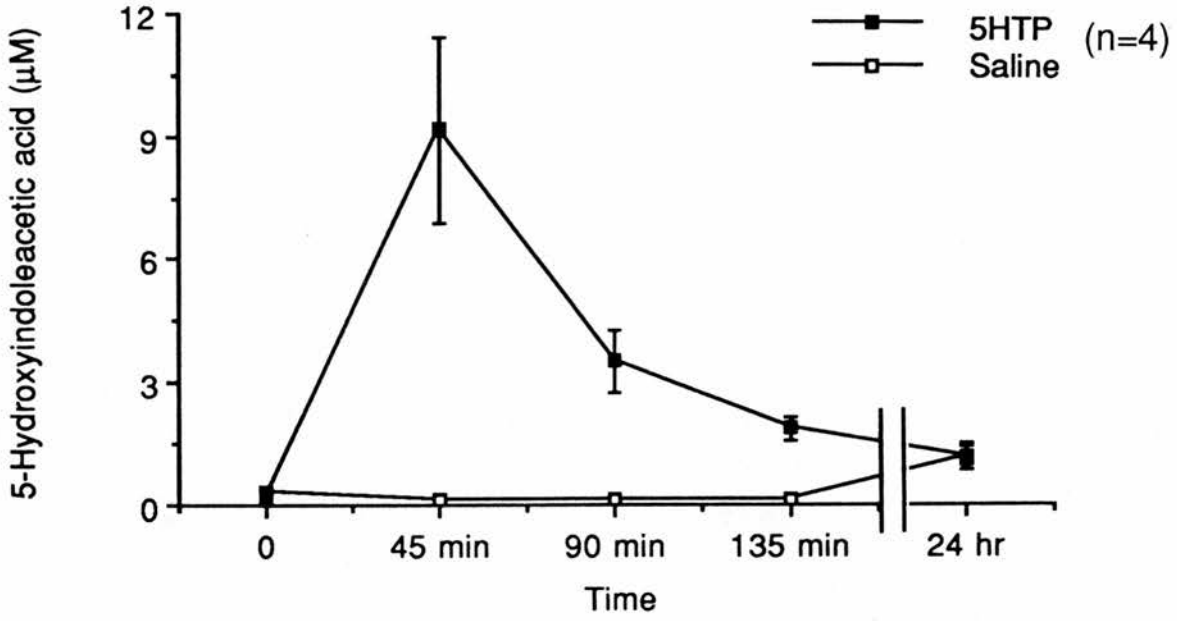


Figure 6.3. **The effect of saline or 5HTP injection on serum 5HIAA concentration in the cannulated rat.**

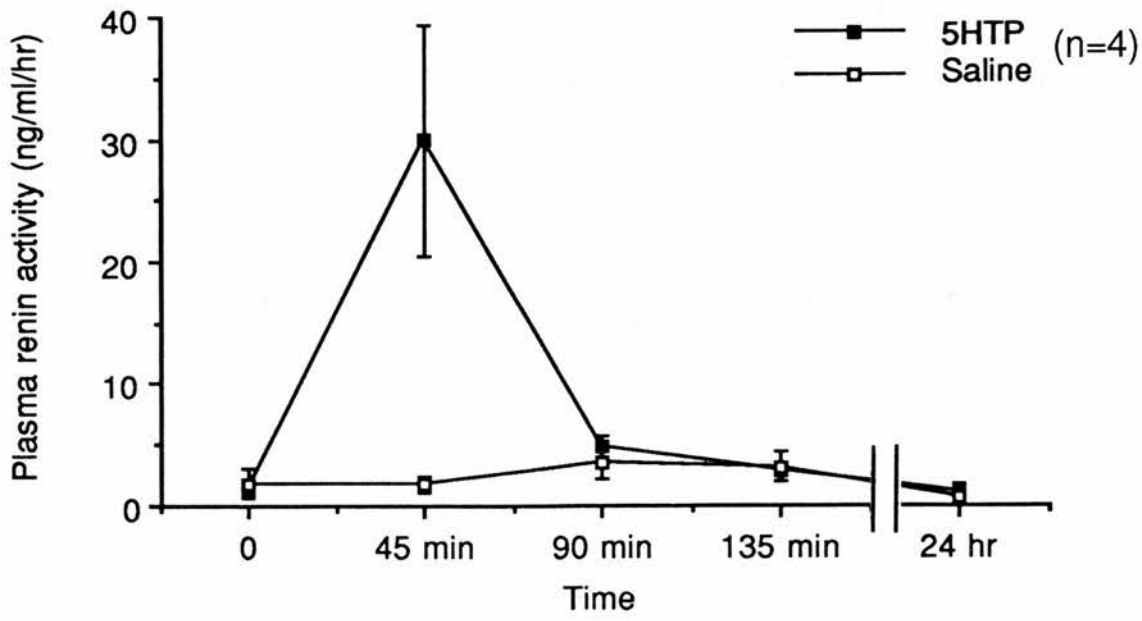


Figure 6.4. **The effect of saline or 5HTP injection on PRA in the cannulated rat.**

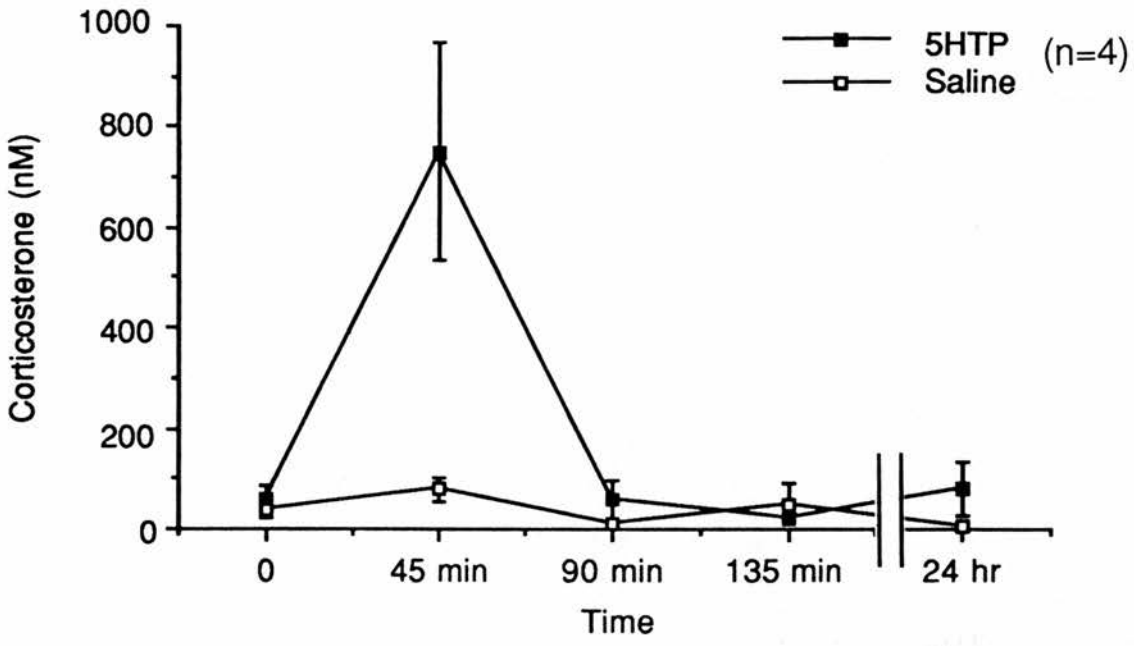


Figure 6.5. **The effect of saline or 5HTP injection on plasma corticosterone concentration in the cannulated rat.**

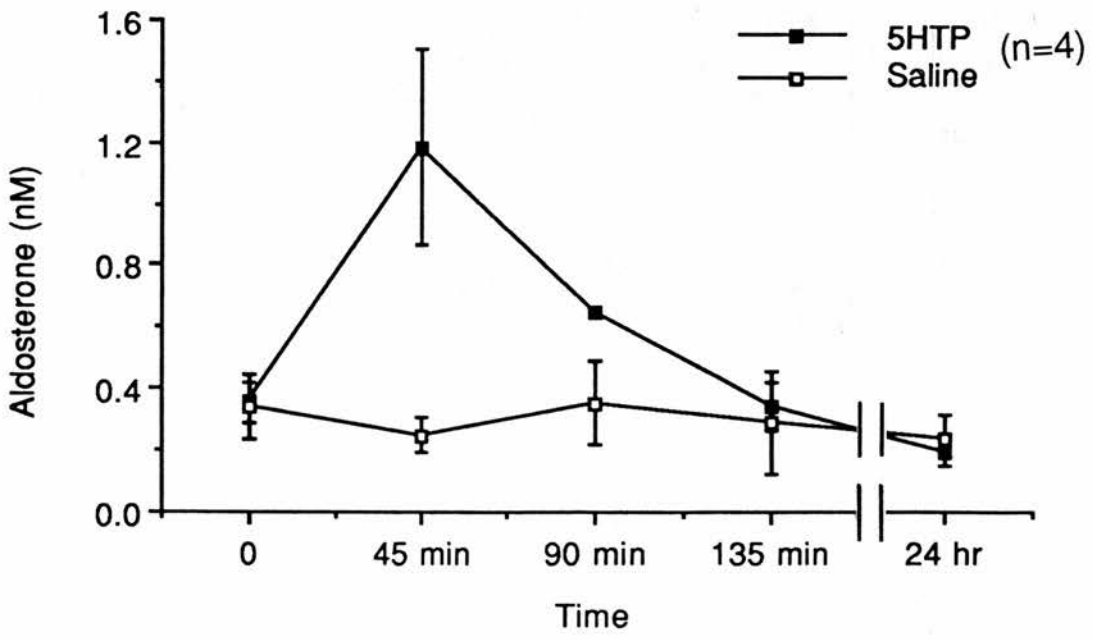


Figure 6.6. **The effect of saline or 5HTP injection on plasma aldosterone concentration in the cannulated rat.**

effect on corticosterone levels.

Figure 6.6 shows the plasma aldosterone concentration in both groups of animals throughout the experimental time course. The basal levels of aldosterone were not significantly different in each group. Following 5HTP administration, aldosterone levels increased maximally from 0.36 ± 0.06 to 1.18 ± 0.32 nM ($p<0.05$) at 45 minutes and returned to basal values within 90 minutes. Administration of saline showed no significant effect on aldosterone levels.

Figure 6.7 shows the plasma sodium concentration in both groups of animals throughout the experimental time course. The basal levels of sodium were not significantly different in each group and there was no significant change following administration of 5HTP or saline.

Figure 6.8 shows the plasma potassium concentration in both groups of animals throughout the experimental time course. The basal levels of potassium were not significantly different in each group and there was no significant change within 45 minutes in either group, although each group showed a general downward trend from the beginning to the end of the study.

6.5.2. Study 2. The effect of 5HTP or saline administration on blood pressure.

It is possible that the hormonal changes observed following 5HTP administration may be secondary to changes in blood pressure caused by serotonin. Serotonin is capable of mediating both vasoconstriction and vaodilatation. A fall in blood pressure would be detected by the juxtaglomerular apparatus of the kidney, resulting in renin release and subsequently angiotensin II, which would not only stimulate aldosterone secretion but also cause

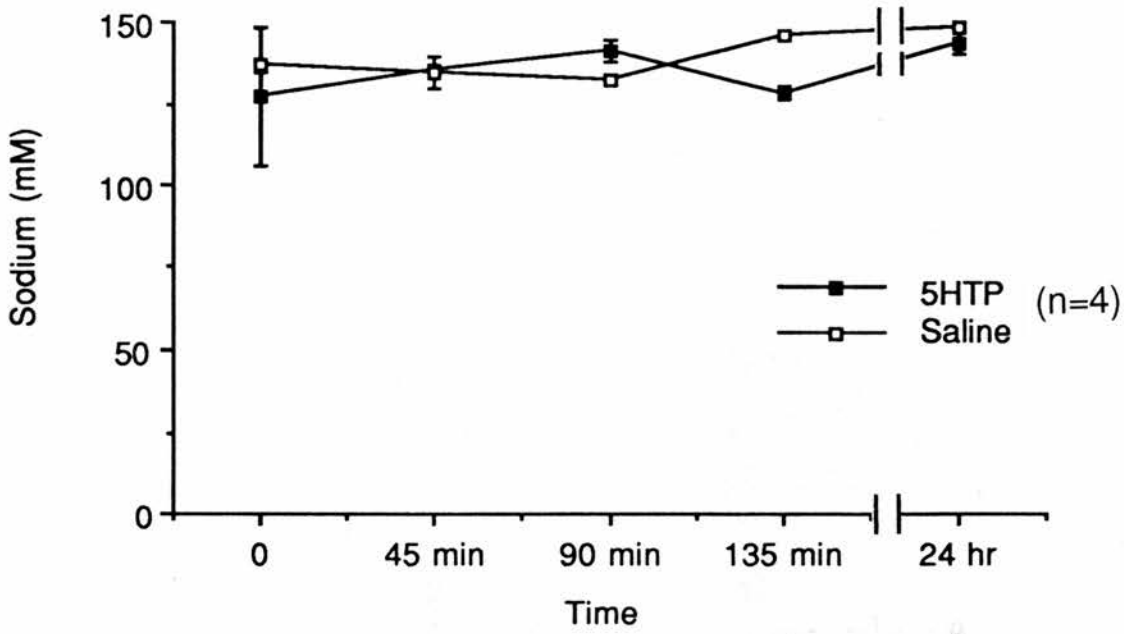


Figure 6.7. **The effect of saline or 5HTP injection on plasma sodium concentration in the cannulated rat.**

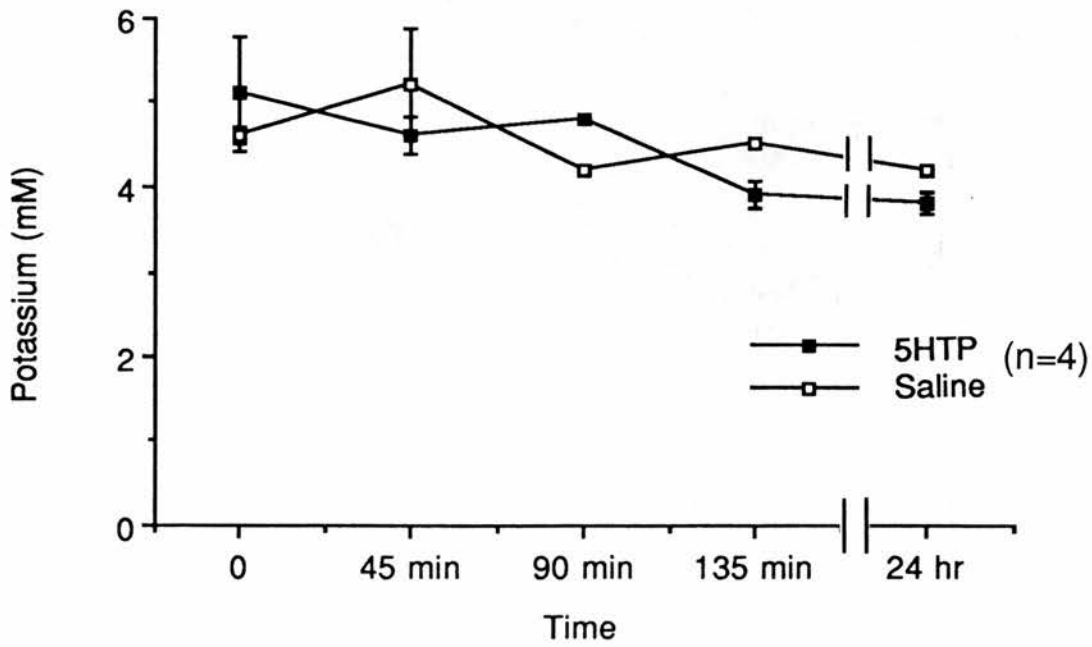


Figure 6.8. **The effect of saline or 5HTP injection on plasma potassium concentration in the cannulated rat.**

vasoconstriction to normalise the falling blood pressure. For these reasons, blood pressure was monitored in two groups of animals every 5 minutes for 45 minutes. One group (n=4) received saline, whilst the other received 5HTP (n=4). Mean arterial blood pressure was calculated from the systolic and diastolic values using the formula described in the materials and methods section 3.4.7.

Figure 6.9 shows the effect of saline or 5HTP administration on mean arterial blood pressure (MABP). Blood pressure was monitored at time=0, before administration of 5HTP or saline, and every 5 minutes thereafter for 45 minutes. Comparison of basal values in the 2 groups revealed a significant ($p<0.05$) difference in blood pressure, which was 112 ± 2.1 mm Hg in the 5HTP treated group and 125 ± 3.2 mm Hg in the saline treated group. Administration of 5HTP caused a significant increase in blood pressure from 112 ± 2.1 to 122 ± 1.5 ($p<0.01$), 124 ± 2.2 ($p<0.01$), 124 ± 3.5 ($p<0.05$), 127 ± 2.0 ($p<0.01$) and 127 ± 2.9 mm Hg ($p<0.01$) at 25, 30, 35, 40 and 45 minutes respectively. Saline administration caused no significant change in blood pressure.

6.5.3. Study 3. The effect of 5HTP or saline administration on dexamethasone and non-dexamethasone pretreated animals.

Having established that administration of 5HTP increased aldosterone secretion, it was necessary to investigate if this was a direct action on the zona glomerulosa, or an indirect effect mediated by activation of the hypothalamo-pituitary adrenal axis, since serotonin is already known to stimulate CRF and ACTH release and also in the preliminary study corticosterone levels increased following 5HTP administration (Weiner and Ganong 1978, Kreiger and Kreiger 1979, Fuller 1981). The possible involvement of the hypothalamo-pituitary adrenal axis was studied by comparing the effect of 5HTP administration on dexamethasone and non-dexamethasone pretreated animals (n=4/group). A third group of animals (n=4), also pretreated with dexamethasone and given saline was also

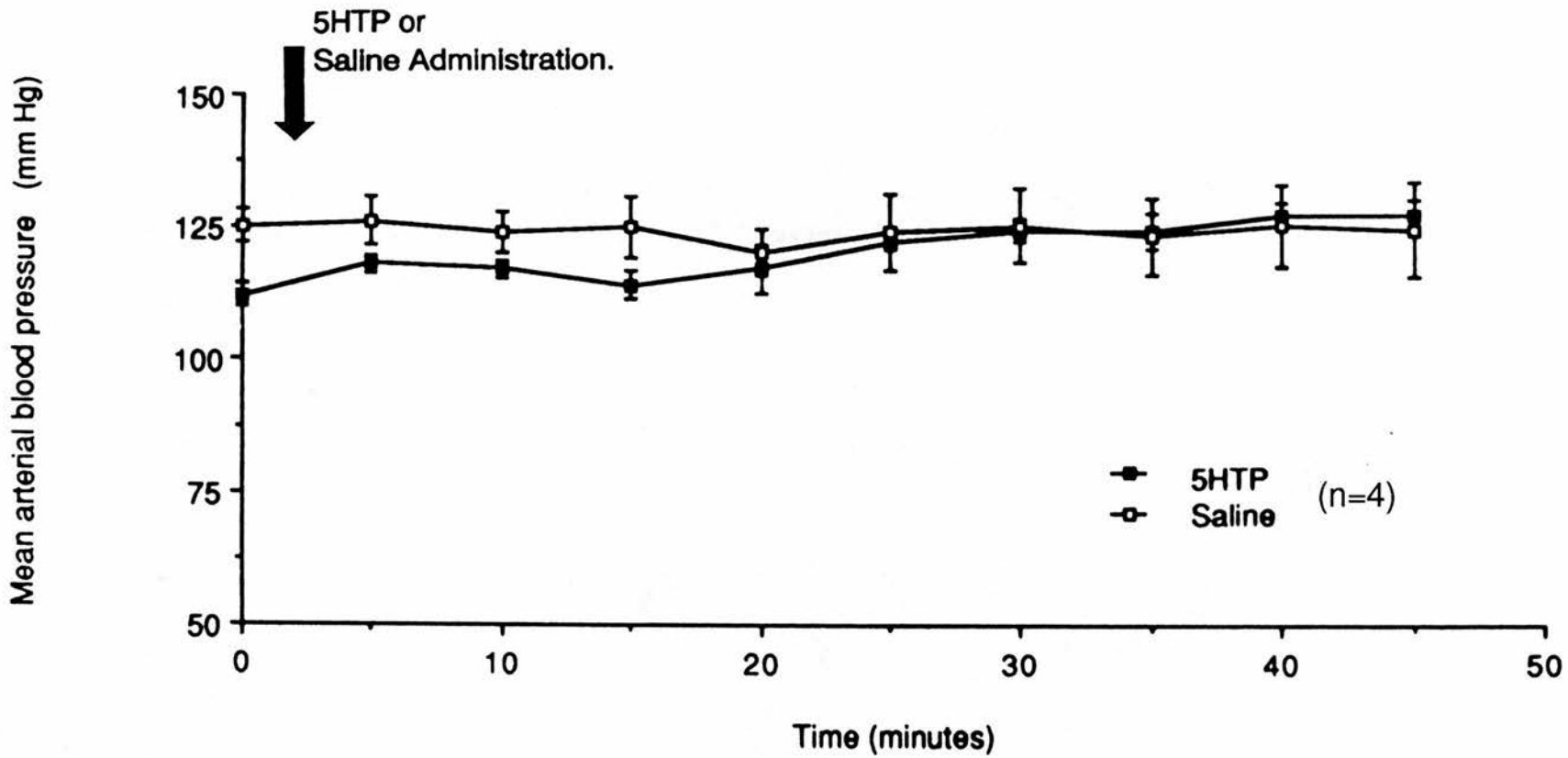


Figure 6.9. **The effect of saline or 5HTP on blood pressure.**

studied. The dexamethasone regime followed has already been described in chapter 3 section 3.4.3.

Figure 6.10 shows serum 5HTP concentration in each of the 3 groups throughout the experimental time course. Basal 5HTP levels in the dexamethasone pretreated groups were not significantly different from the untreated group. Following 5HTP administration, 5HTP levels increased maximally at 45 minutes from 2.1 $\pm$ 0.9 to 16.9 $\pm$ 1.7 μM ($p<0.001$) in the dexamethasone pretreated group and from 1.4 $\pm$ 0.1 to 21.9 $\pm$ 0.8 μM ($p<0.001$) in the untreated group. Both returned to basal values within 135 minutes. Comparison of the increases observed at 45 minutes revealed that dexamethasone pretreatment had no significant effect on 5HTP uptake or clearance following its administration. Administration of saline to dexamethasone pretreated animals showed no significant effect on 5HTP levels.

Figure 6.11 shows serum serotonin concentration in each of the 3 groups throughout the experimental time course. Basal serotonin levels in the dexamethasone pretreated groups were not significantly different from the untreated group. Following 5HTP administration, serotonin levels increased maximally from 1.6 $\pm$ 0.4 to 3.1 $\pm$ 0.5 μM ($p<0.05$) at 90 minutes in the dexamethasone pretreated group and from 1.3 $\pm$ 0.3 to 4.8 $\pm$ 0.6 μM ($p<0.01$) at 45 minutes in the untreated group. Both returned to basal values within 24 hours. Although there was no significant difference in the maximum levels of serotonin following 5HTP administration, the time course of the increase was slightly altered. However, comparison of the increase observed at 45 minutes in each group also showed no significant difference. Administration of saline to dexamethasone pretreated animals showed no significant effect on serotonin levels.

Figure 6.12 shows serum 5HIAA concentration in each of the 3 groups throughout the experimental time course. Basal 5HIAA levels in the dexamethasone pretreated groups were

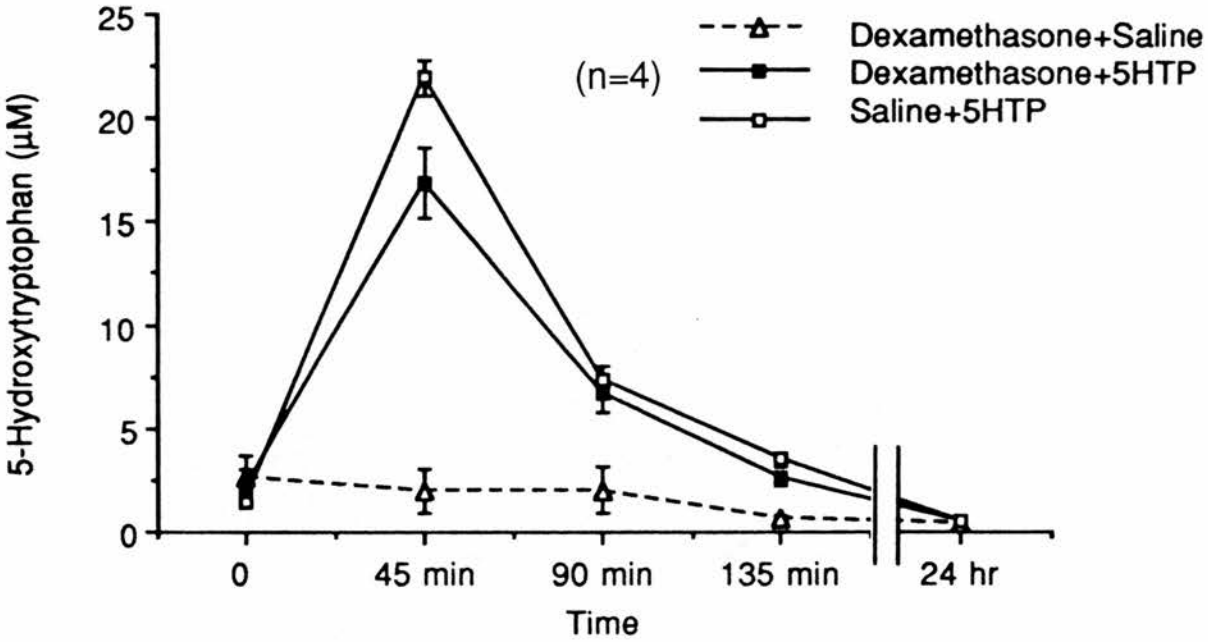


Figure 6.10. **The effect of saline or 5HTP on serum 5HTP concentration in the cannulated rat pretreated with saline or dexamethasone.**

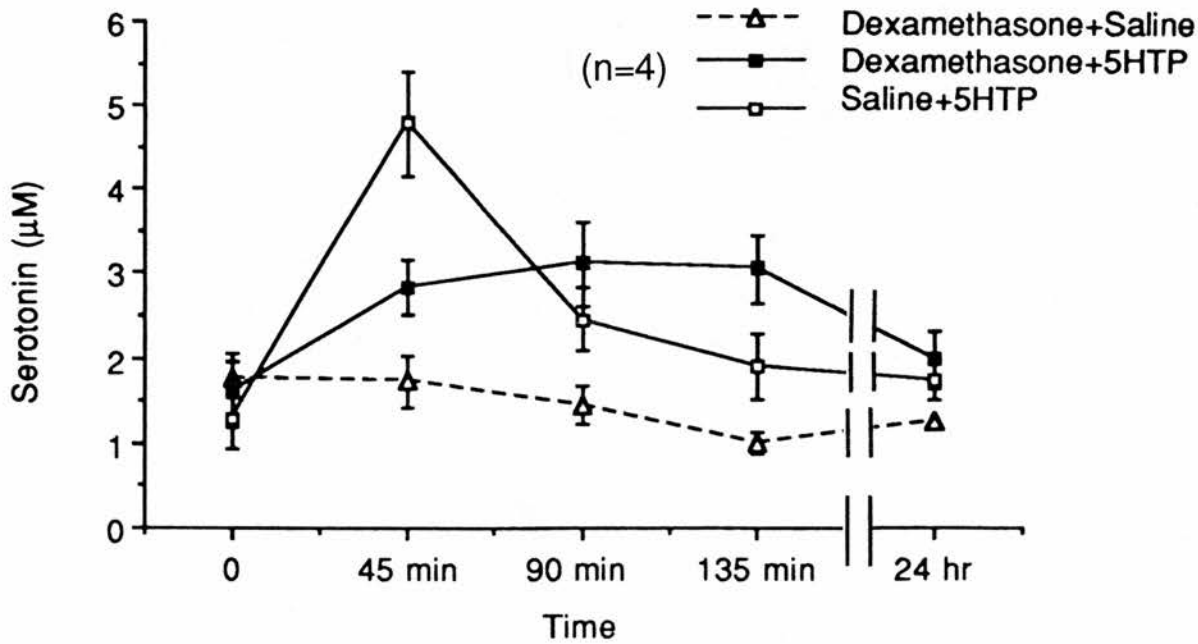


Figure 6.11. **The effect of saline or 5HTP on serum serotonin concentration in the cannulated rat pretreated with saline or dexamethasone.**

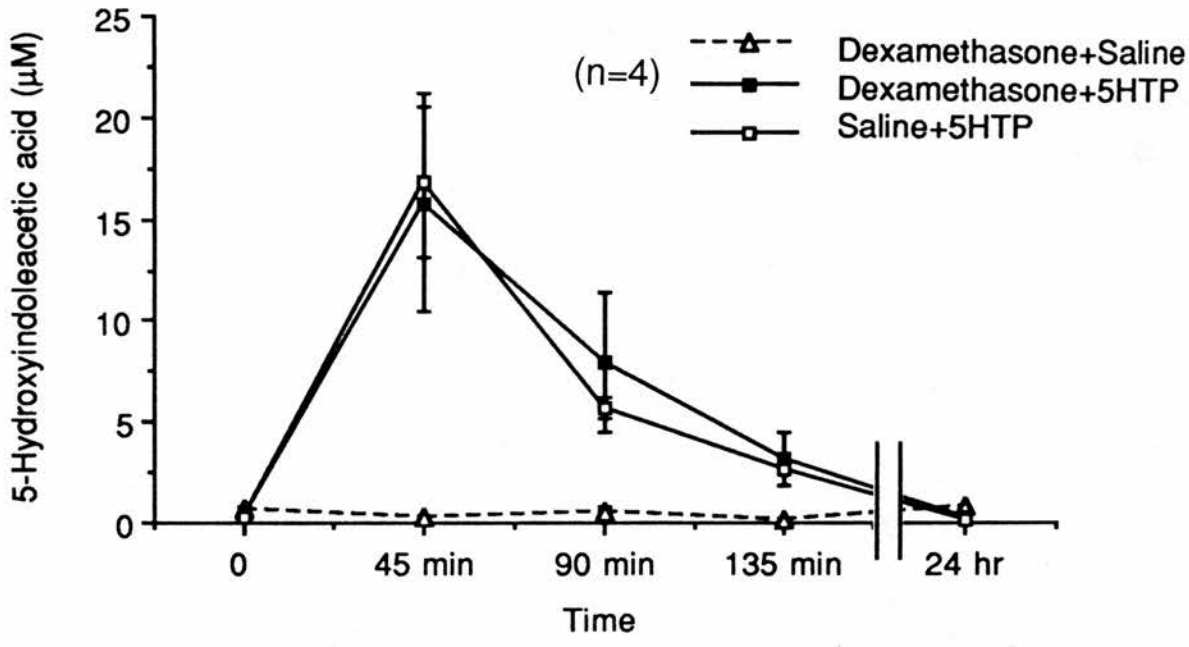


Figure 6.12. **The effect of saline or 5HTP on serum 5HIAA concentration in the cannulated rat pretreated with saline or dexamethasone.**

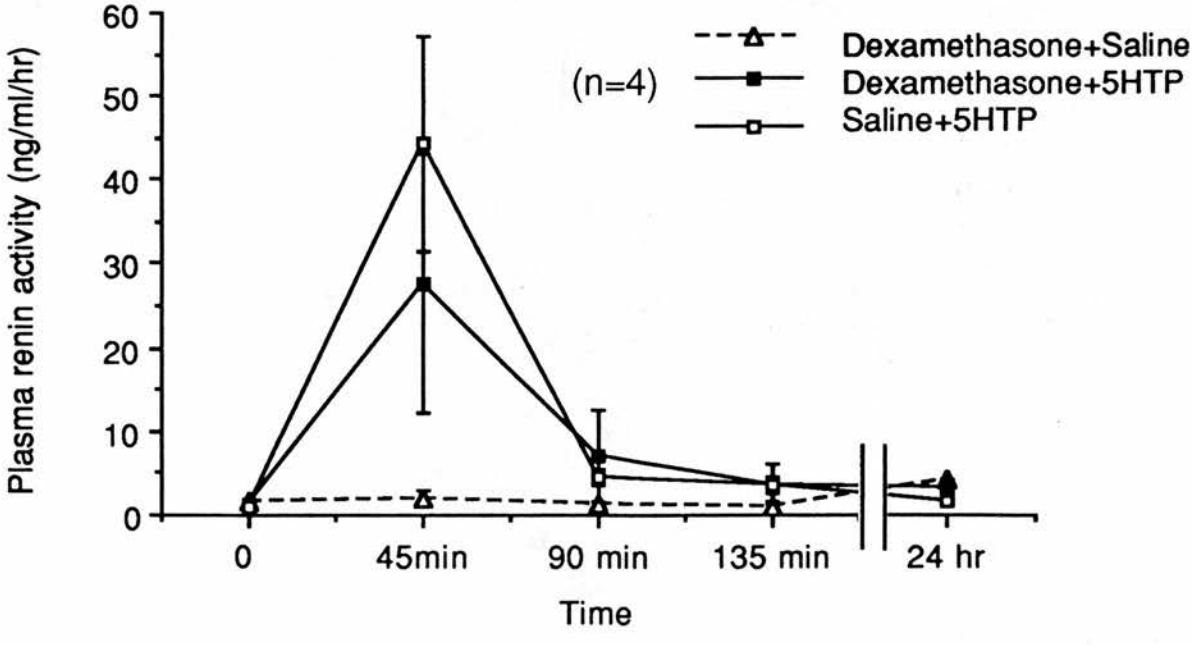


Figure 6.13. **The effect of saline or 5HTP on PRA in the cannulated rat pretreated with saline or dexamethasone.**

not significantly different from the untreated group. Following 5HTP administration, 5HIAA levels increased maximally at 45 minutes from 0.3 ± 0.05 to 15.8 ± 5.3 μM ($p<0.01$) in the dexamethasone pretreated group and from 0.3 ± 0.04 to 16.8 ± 3.7 μM ($p<0.05$) in the untreated group. Both returned to basal values within 24 hours. Comparison of the increases observed at 45 minutes revealed that dexamethasone had no significant effect on the formation of 5HIAA following administration of 5HTP. Administration of saline to captopril pretreated animals showed no significant effect on 5HIAA levels.

Figure 6.13 shows PRA in each of the 3 groups throughout the experimental time course. Basal PRA in the dexamethasone pretreated groups were not significantly different from the untreated group. Following 5HTP administration, PRA increased maximally at 45 minutes from 1.2 ± 0.5 to 27.6 ± 15.4 ng/ml/hr ($p<0.05$) in the dexamethasone pretreated group and from 0.9 ± 0.2 to 44.3 ± 12.8 ng/ml/hr ($p<0.05$) in the untreated group. Both returned to basal values within 135 minutes. Comparison of the increases observed at 45 minutes revealed that dexamethasone had no significant effect on PRA following administration of 5HTP. Administration of saline to dexamethasone pretreated animals showed no significant effect on PRA.

Figure 6.14 shows plasma corticosterone concentration in each of the 3 groups throughout the experimental time course. Basal corticosterone levels in the dexamethasone pretreated groups were 20.5 ± 12.9 nM and 41.9 ± 38.7 compared with 99.4 ± 28.7 in the non-dexamethasone treated group. Following 5HTP administration, corticosterone levels increased maximally from 99.4 ± 28.7 to 762.5 ± 147.4 nM ($p<0.01$) at 45 minutes in the untreated group and returned to basal values within 90 minutes. There was no significant increase in corticosterone in the dexamethasone pretreated group following 5HTP administration. Comparison of the levels of corticosterone at 45 minutes in the 5HTP treated groups revealed that dexamethasone pretreatment significantly inhibited the formation of

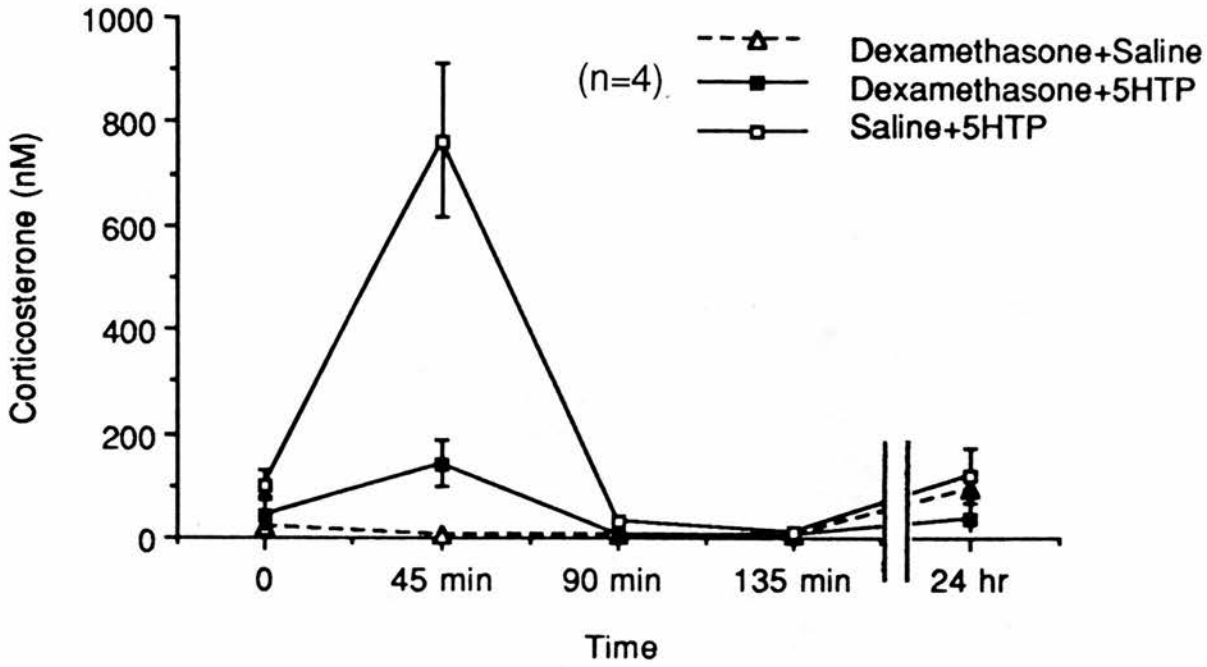


Figure 6.14. **The effect of saline or 5HTP on plasma corticosterone concentration in the cannulated rat pretreated with saline or dexamethasone.**

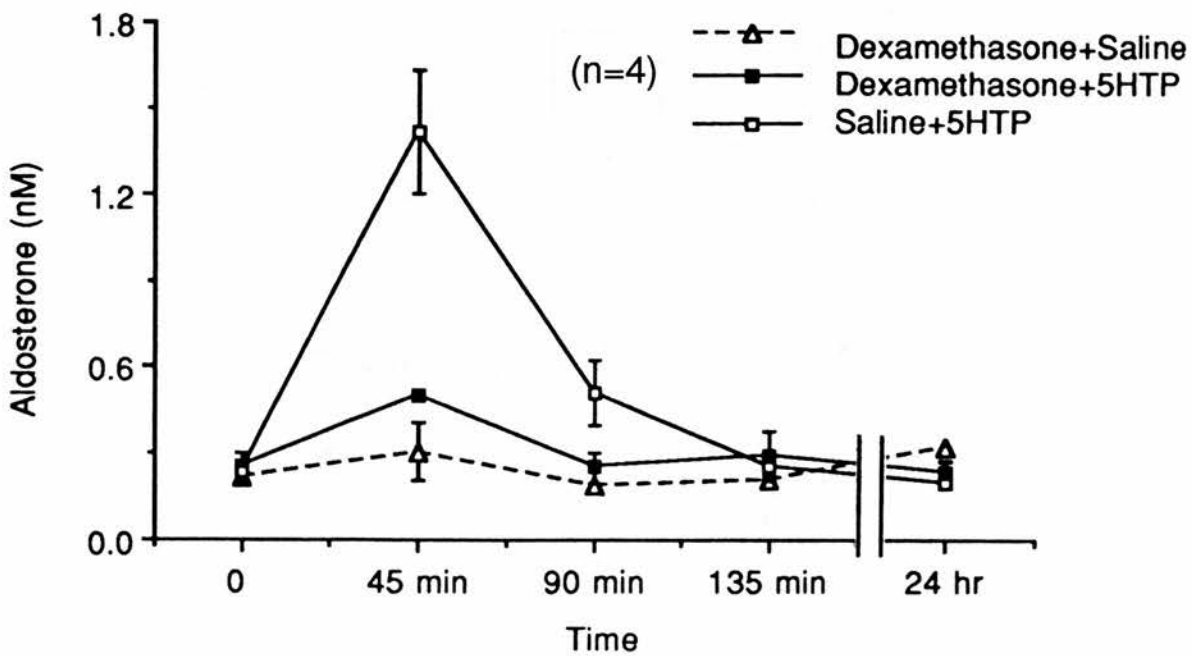


Figure 6.15. **The effect of saline or 5HTP on plasma aldosterone concentration in the cannulated rat pretreated with saline or dexamethasone.**

corticosterone following administration of 5HTP. Administration of saline to dexamethasone pretreated animals showed no significant effect on corticosterone levels.

Figure 6.15 shows plasma aldosterone concentration in each of the 3 groups throughout the experimental time course. Basal aldosterone levels in the dexamethasone pretreated groups were not significantly different from the untreated group. Following 5HTP administration, aldosterone levels increased maximally at 45 minutes from 0.25 $\pm$ 0.05 to 0.50 $\pm$ 0.01 nM ($p<0.01$) in the dexamethasone pretreated group and from 0.24 $\pm$0.01 to 1.42 $\pm$ 0.21 nM ($p<0.001$) in the untreated group. Both returned to basal values within 135 minutes. Comparison of the increases observed at 45 minutes revealed that dexamethasone pretreatment significantly inhibited aldosterone secretion following administration of 5HTP ($p<0.01$). Administration of saline to dexamethasone pretreated animals showed no significant effect on aldosterone levels.

Figure 6.16 shows plasma sodium concentration in each of the 3 groups throughout the experimental time course. Basal sodium levels in the dexamethasone pretreated groups were not significantly different from the untreated group. There was no significant change in sodium levels in any of the groups at any time point following administration of either 5HTP or saline.

Figure 6.17 shows plasma potassium concentration in each of the 3 groups throughout the experimental time course. There was a significant decrease in potassium levels in all 3 groups within 45 minutes. Following 5HTP administration, potassium decreased from 6.6 $\pm$ 0.2 to 6.0 $\pm$ 0.2 mM in the dexamethasone pretreated group and from 7.6 $\pm$ 1.3 to 5.8 $\pm$ 0.1 mM in the untreated group. Potassium levels in the dexamethasone pretreated group receiving saline decreased from 6.6 $\pm$ 0.2 to 5.5 $\pm$ 0.1 mM. All 3 groups showed a general downward trend thereafter.

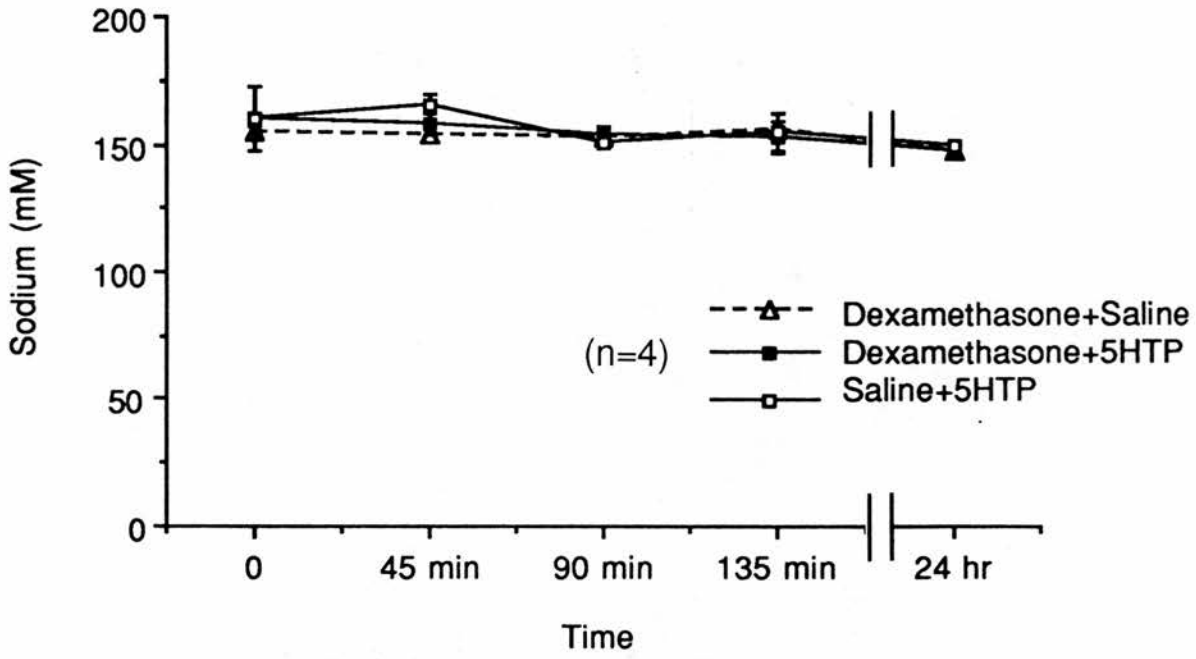


Figure 6.16. **The effect of saline or 5HTP on plasma sodium concentration in the cannulated rat pretreated with saline or dexamethasone.**

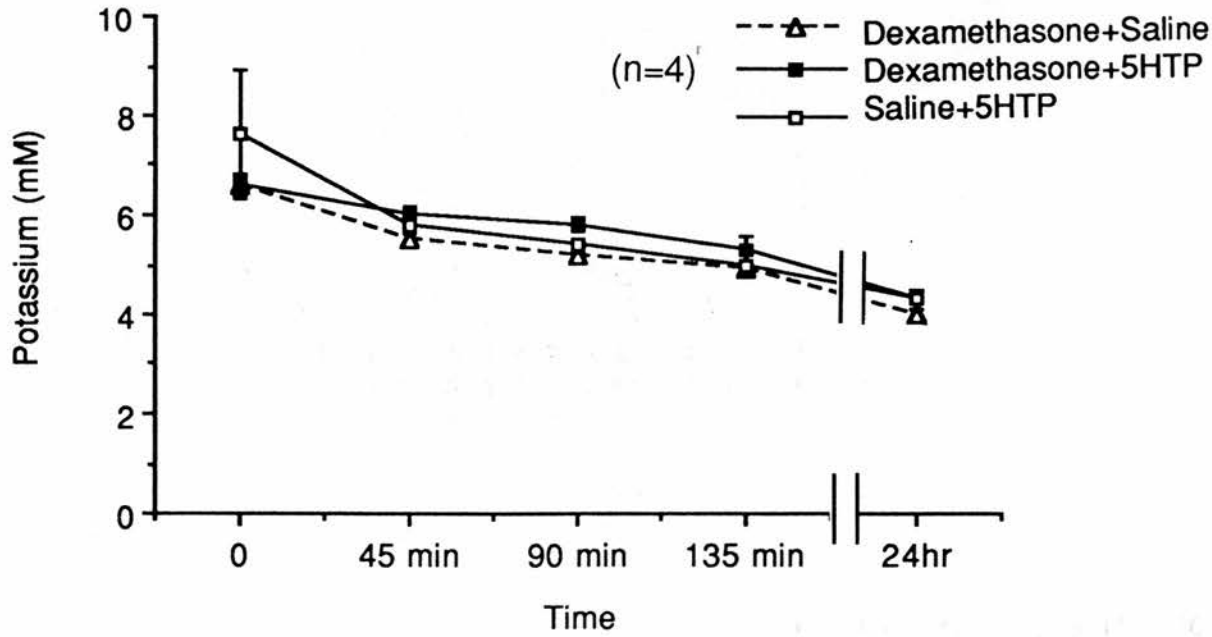


Figure 6.17. **The effect of saline or 5HTP on plasma potassium concentration in the cannulated rat pretreated with saline or dexamethasone.**

6.5.4. Study 4. The effect of 5HTP or saline administration on captopril and non-captopril pretreated animals

The results of the previous section suggest that the stimulatory effect of 5HTP on aldosterone secretion is dependent upon an intact hypothalamo-pituitary adrenal axis. However, it is also necessary to consider the role of the renin-angiotensin system, the major physiological regulator of aldosterone secretion, as the increase in PRA following administration of 5HTP in the preliminary study suggests that this is also activated.

Therefore, the effect of 5HTP administration was studied in a group of animals (n=4) pretreated for 1 week with the ACE inhibitor captopril and compared with a non-pretreated group also given 5HTP. A control group (n=4) pretreated with captopril and injected with saline instead of 5HTP was also studied. Captopril effectively blocks formation of angiotensin II, which increases aldosterone secretion. Hence any activation of the renin-angiotensin system by 5HTP would be blocked and aldosterone secretion inhibited. The captopril regime followed has already been described in chapter 3 section 3.4.2.

Figure 6.18 shows serum 5HTP concentration in each of the 3 groups throughout the experimental time course. Basal 5HTP levels in the captopril pretreated groups were not significantly different from the untreated group. Following 5HTP administration, 5HTP levels increased maximally at 45 minutes from 1.5 ± 0.5 to 14.3 ± 0.9 μM ($p<0.01$) in the captopril pretreated group and from 0.6 ± 0.2 to 18.6 ± 1.0 μM ($p<0.001$) in the untreated group. Both returned to basal values within 135 minutes. Comparison of the increases in each group at 45 minutes revealed that captopril pretreatment had a very small but significant inhibitory effect on the uptake of 5HTP ($p<0.05$). Administration of saline to captopril pretreated animals showed no significant effect on 5HTP levels.

Figure 6.19 shows serum serotonin concentration in each of the 3 groups throughout the experimental time course. Basal serotonin levels in the captopril pretreated groups were

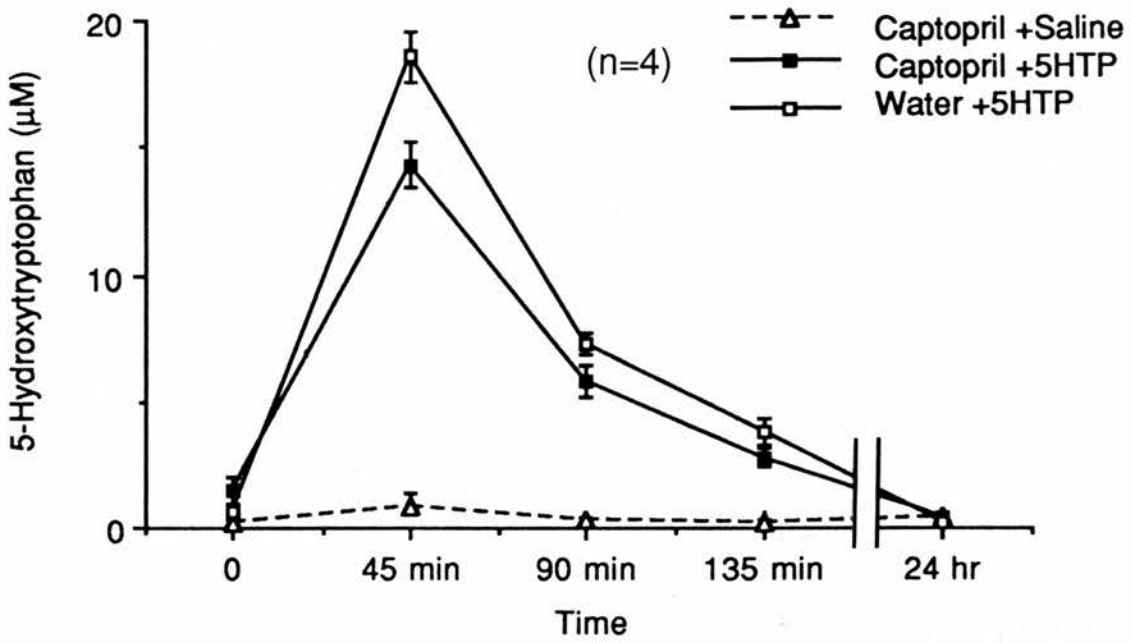


Figure 6.18. **The effect of saline or 5HTP on serum 5HTP concentration in the cannulated rat pretreated with or without captopril.**

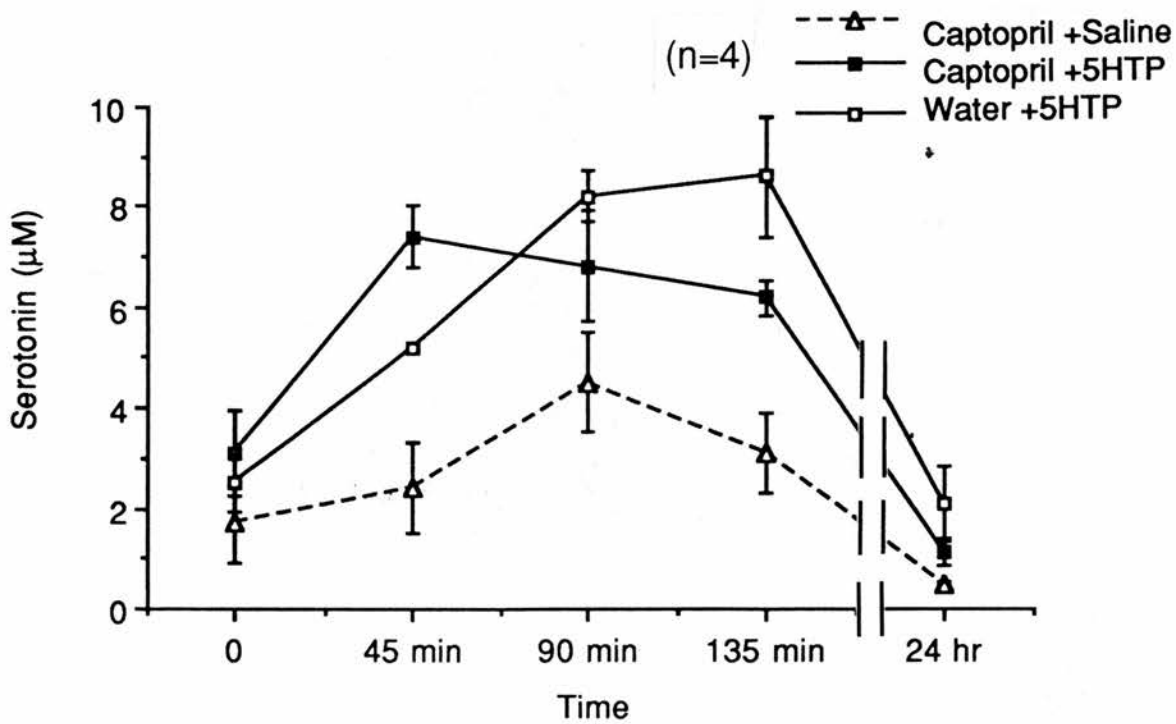


Figure 6.19. **The effect of saline or 5HTP on serum serotonin concentration in the cannulated rat pretreated with or without captopril.**

not significantly different from the untreated group. Following 5HTP administration, serotonin levels increased maximally from 3.1 ± 0.9 to 7.4 ± 0.6 μM ($p<0.05$) at 45 minutes in the captopril pretreated group and from 2.5 ± 0.6 to 8.2 ± 0.5 μM ($p<0.01$) at 135 minutes in the untreated group. The increase observed at 45 minutes in this group was also significant ($p<0.05$), though not maximal. Both returned to basal values within 24 hours. Although there was no significant difference in the maximal levels of serotonin following 5HTP administration in each group, the time couse of the increase was slightly altered and comparison of the increases at 45 minutes revealed that captopril pretreatment significantly enhanced formation of serotonin ($p<0.05$).

Figure 6.20 shows serum 5HIAA concentration in each of the 3 groups throughout the experimental time course. Basal 5HIAA levels in the captopril pretreated groups were not significantly different from the untreated group. Following 5HTP administration, 5HIAA levels increased maximally at 45 minutes from 0.8 ± 0.7 to 25.4 ± 4.8 μM ($p<0.05$) in the captopril pretreated group and from 0.2 ± 0.1 to 28.9 ± 2.9 μM ($p<0.001$) in the untreated group. Both returned to basal values within 24 hours. Comparison of the increases in each group at 45 minutes revealed that captopril pretreatment had no significant effect on the formation of 5HIAA following 5HTP administration. Administration of saline to captopril pretreated animals showed no significant effect on 5HIAA levels.

Figure 6.21 shows PRA in each of the 3 groups throughout the experimental time course. Basal PRAs were 10.7 ± 2.0 ($p<0.01$) and 10.9 ± 7.7 ng/ml/hr (NS) in each of the captopril pretreated groups compared with 2.4 ± 1.0 ng/ml/hr in the non-captopril pretreated group. Following 5HTP administration, PRA increased maximally at 45 minutes from 10.9 ± 7.7 to 137.3 ± 19.2 ng/ml/hr ($p<0.05$) in the captopril pretreated group and from 2.4 ± 1.0 to 67.4 ± 21.3 ng/ml/hr ($p<0.05$) in the untreated group. Both returned to basal values within 24 hours. Although basal PRA is significantly increased, comparison of the increase in each

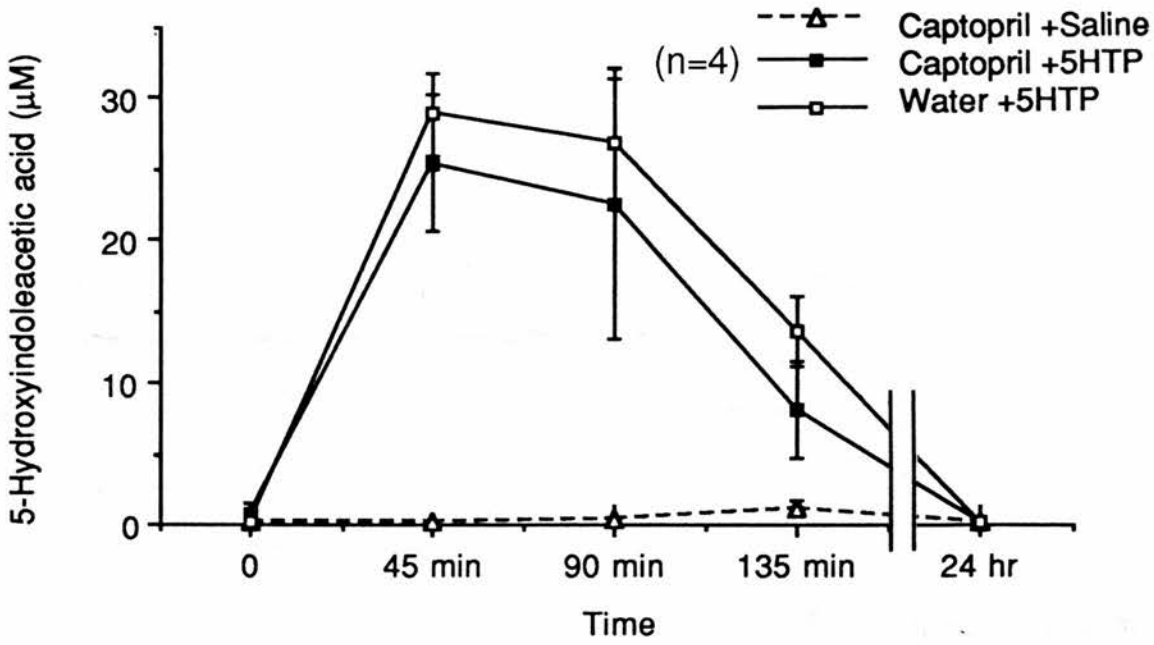


Figure 6.20. **The effect of saline or 5HTP on serum 5HIAA concentration in the cannulated rat pretreated with or without captopril.**

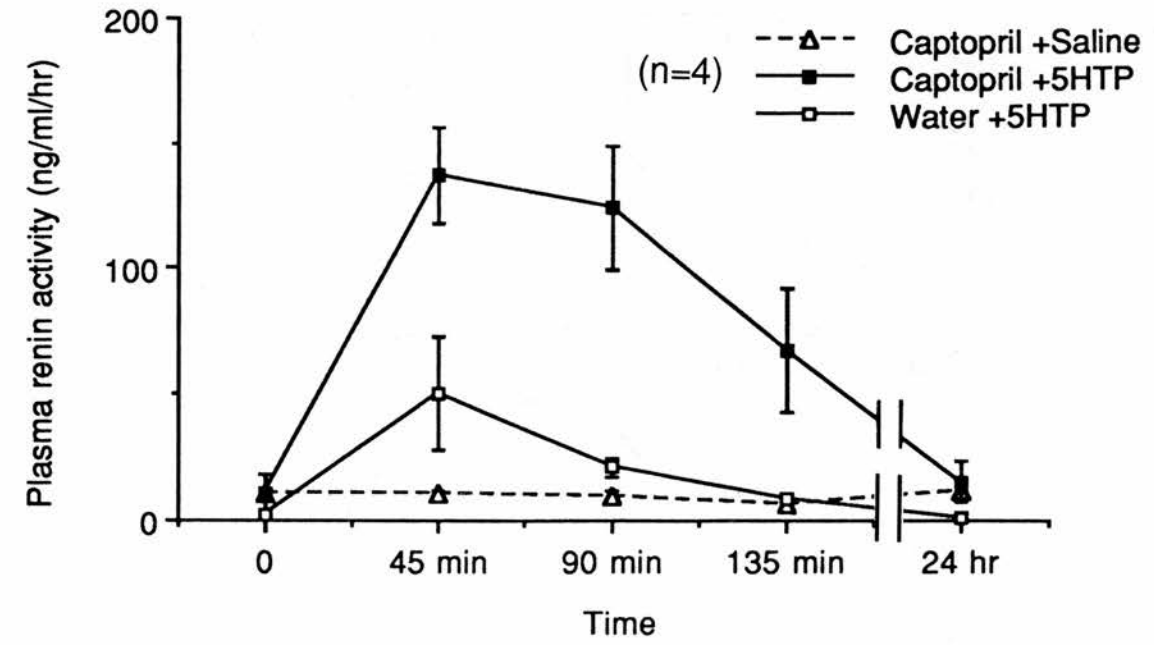


Figure 6.21. **The effect of saline or 5HTP on PRA in the cannulated rat pretreated with or without captopril.**

group at 45 minutes revealed that captopril pretreatment had no significant effect on the increase in PRA following 5HTP administration. However, the time course of the return to basal levels was considerably prolonged in the captopril pretreated group. Administration of saline to captopril pretreated animals showed no significant effect on PRA.

Figure 6.22 shows plasma corticosterone concentration in each of the 3 groups throughout the experimental time course. Basal corticosterone levels in the captopril pretreated groups were not significantly different from the untreated group. Following 5HTP administration, corticosterone levels increased maximally at 45 minutes from 92.6 $\pm$ 28.4 to 842.4 $\pm$ 62.8 nM ($p<0.001$) in the captopril pretreated group and from 48.7 $\pm$ 15.5 to 791 $\pm$ 90.8 nM ($p<0.01$) in the untreated group. Both returned to basal values within 135 minutes. Comparison of the increases in each group at 45 minutes revealed that captopril pretreatment had no significant effect on the formation of corticosterone following 5HTP administration. Administration of saline to captopril pretreated animals showed no significant effect on corticosterone levels.

Figure 6.23 shows plasma aldosterone concentration in each of the 3 groups throughout the experimental time course. Basal aldosterone levels in the captopril pretreated groups were not significantly different from the untreated group. Following 5HTP administration, aldosterone levels increased maximally at 90 minutes from 0.34 $\pm$ 0.04 to 0.65 $\pm$ 0.02 nM ($p<0.05$) in the captopril pretreated group and from 0.38 $\pm$ 0.02 to 2.0 $\pm$ 0.56 nM ($p<0.05$) in the untreated group. Both returned to basal values within 24 hours. Comparison of the increases in each group at 90 minutes revealed that captopril pretreatment significantly inhibited aldosterone secretion following 5HTP administration ($p<0.05$). Administration of saline to captopril pretreated animals showed no significant effect on aldosterone levels.

Figure 6.24 shows plasma sodium concentration in each of the 3 groups throughout

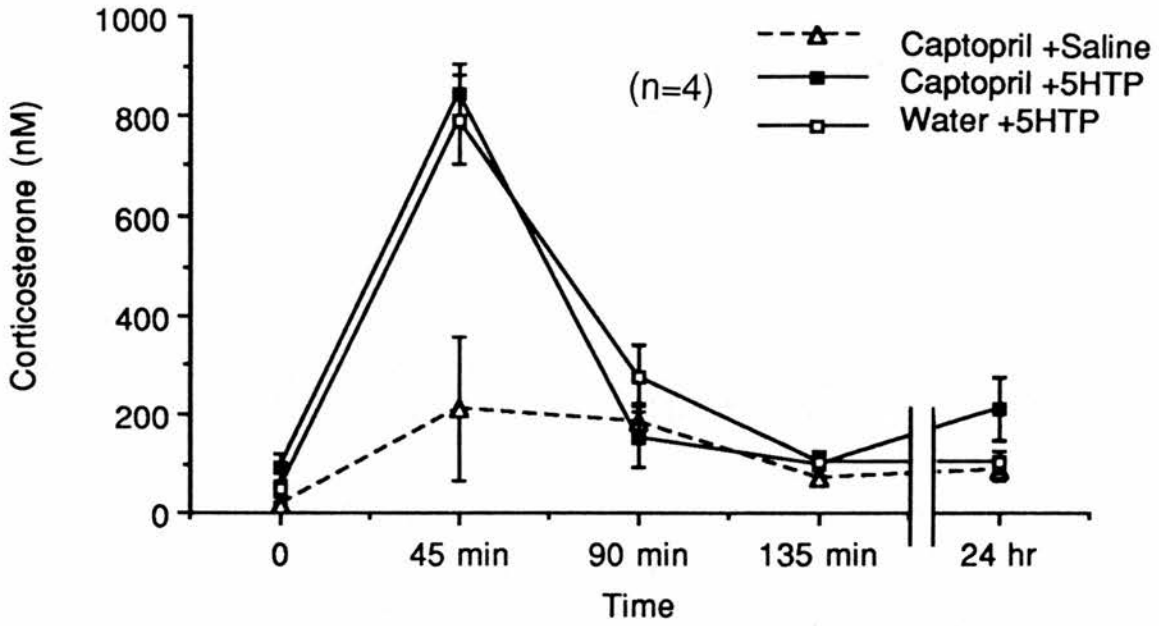


Figure 6.22. **The effect of saline or 5HTP on plasma corticosterone concentration in the cannulated rat pretreated with or without captopril.**

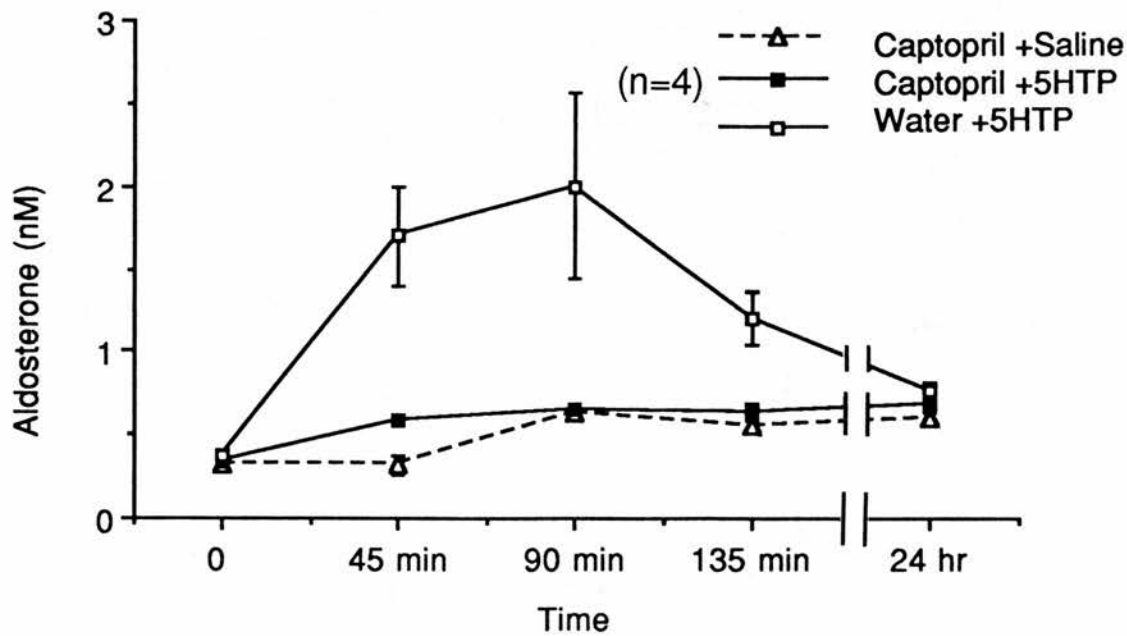


Figure 6.23. **The effect of saline or 5HTP on plasma aldosterone concentration in the cannulated rat pretreated with or without captopril.**

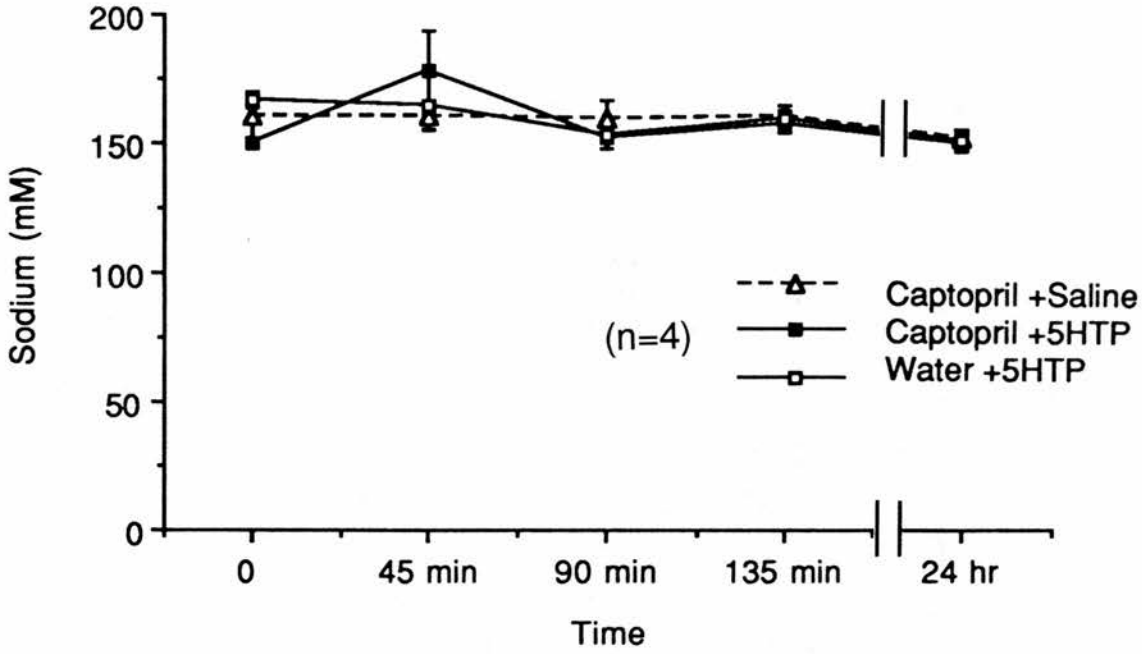


Figure 6.24. **The effect of saline or 5HTP on plasma sodium concentration in the cannulated rat pretreated with or without captopril.**

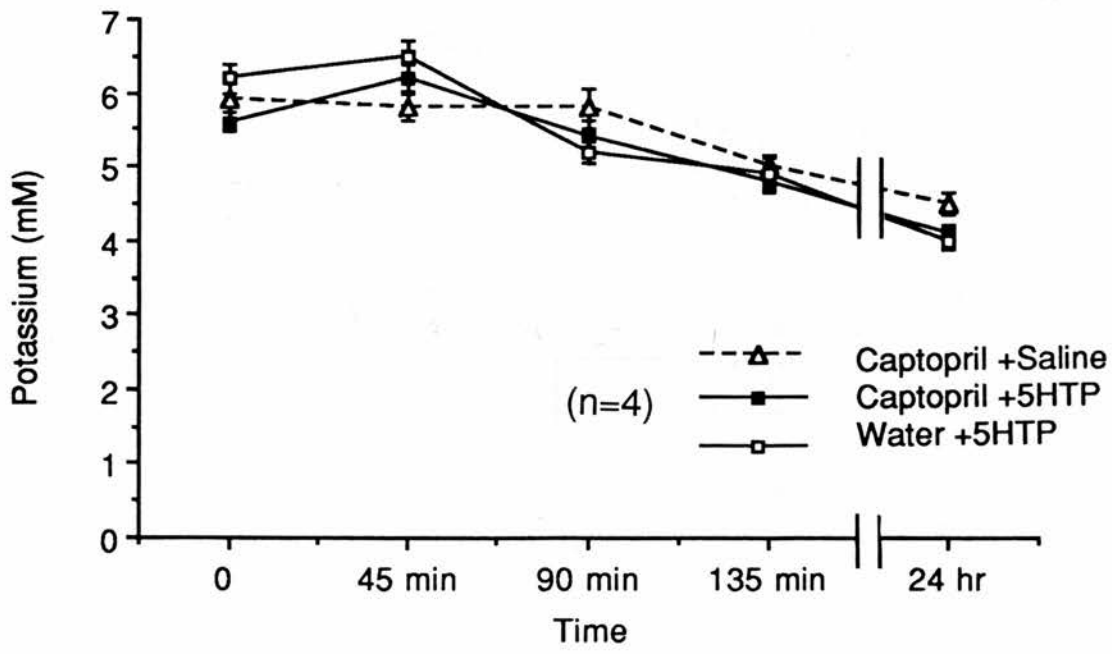


Figure 6.25. **The effect of saline or 5HTP on plasma potassium concentration in the cannulated rat pretreated with or without captopril.**

the experimental time course. Basal sodium levels in the captopril pretreated groups were 160 ± 7.4 (NS) and 150 ± 1.6 mM ($p<0.01$) compared to 166.8 ± 3 mM in the untreated control group. There was no significant change in sodium levels in any of the groups at any time point following administration of either 5HTP or saline.

Figure 6.25 shows plasma potassium concentration in each of the 3 groups throughout the experimental time course. Basal potassium levels in the captopril pretreated groups were 5.9 ± 0.2 (NS) and 5.6 + 0.1 mM ($p<0.05$) compared to 6.2 ± 0.2 mM in the untreated control group. Potassium levels showed no significant change within 45 minutes in any of the groups. However, the levels in all 3 groups showed a general downward trend from the beginning to the end of the experiment.

6.5.5. Study 5. The permissive requirement of angiotensin II in the action of 5HTP

The previous study using captopril illustrates quite clearly that the renin-angiotensin system is involved to some degree in the action of 5HTP. However, it fails to clarify if the effects of 5HTP are mediated by increases in circulating angiotensin II itself, or whether it acts purely in a permissive capacity, and is required to maintain normal adrenal function. In order to investigate this hypothesis the effect of 5HTP administration has been compared in untreated animals (n=4), those pretreated with captopril (n=5) as before, and also in a third group (n=4), pretreated with captopril but supplemented with normal circulating levels of angiotensin II administered by osmotic mini-pump. In effect, the treatment with captopril plus angiotensin II continues to block activation of the renin-angiotensin system by serotonin. However, normal adrenal function is maintained by the presence of the angiotensin II.

Figure 6.26 shows serum 5HTP concentration in each of the 3 groups throughout the experimental time course. There was no significant difference in basal levels of 5HTP

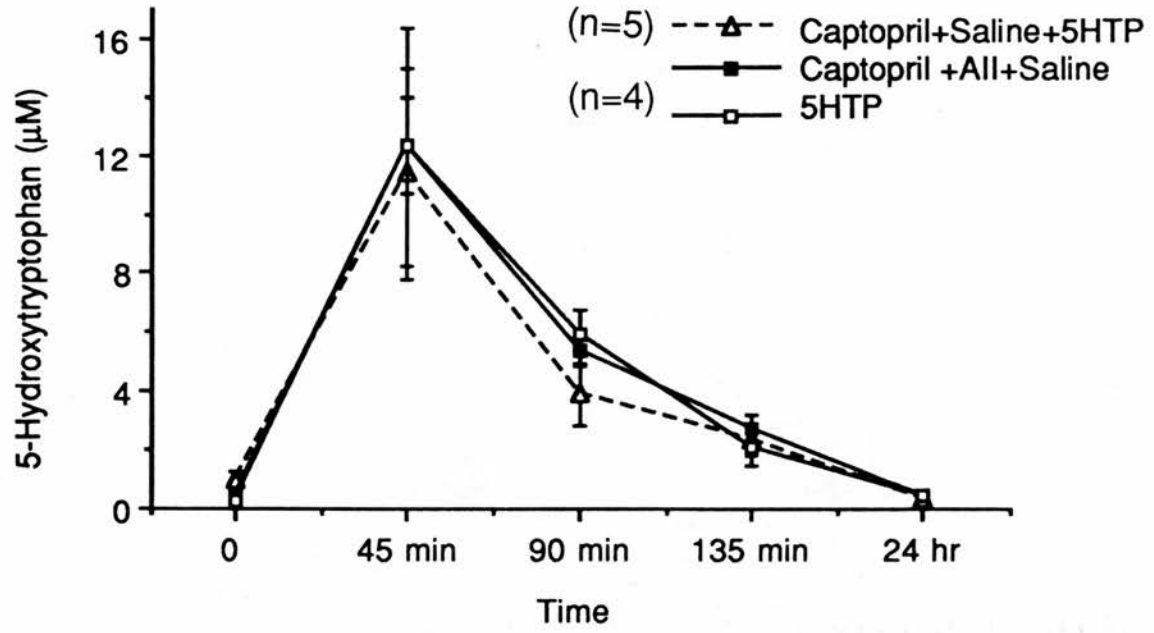


Figure 6.26. **The effect of 5HTP injection on serum 5HTP concentration in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

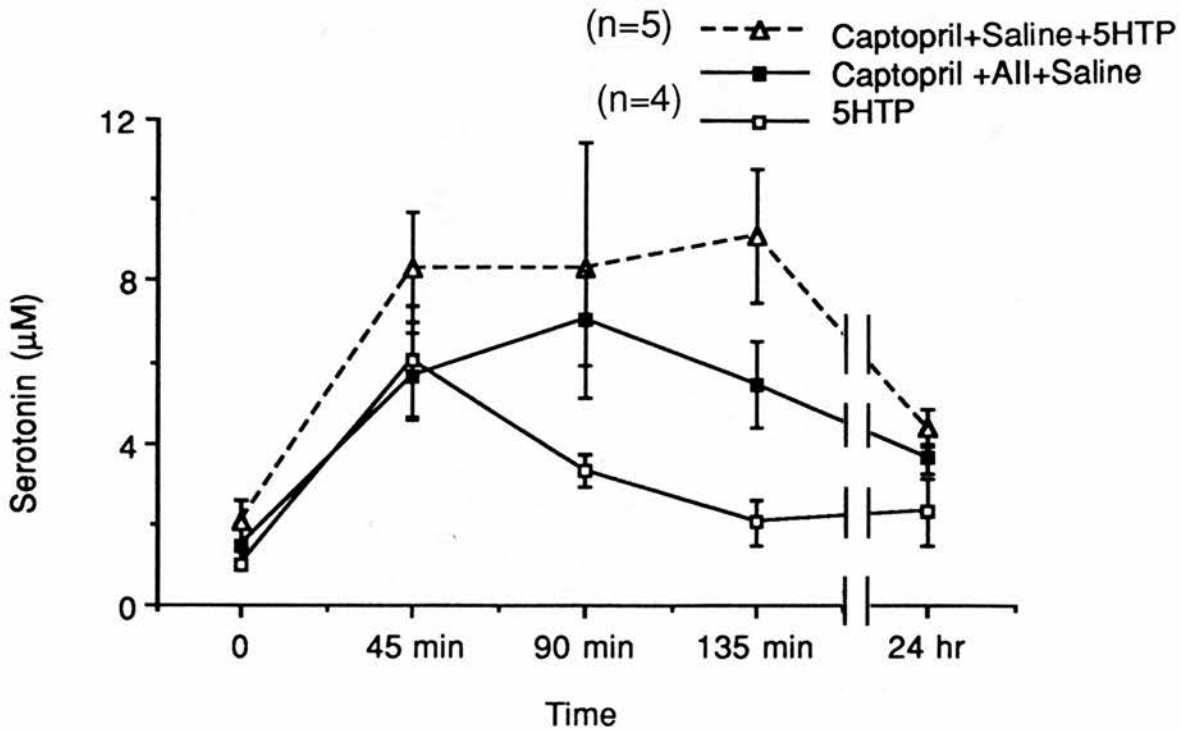


Figure 6.27. **The effect of 5HTP injection on serum serotonin concentration in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

between the 3 groups. Following 5HTP administration, 5HTP increased maximally at 45 minutes from 1.0 ± 0.3 to 11.4 ± 3.6 μM ($p<0.05$) in the captopril pretreated group, from 0.4 ± 0.1 to 12.3 ± 1.7 μM ($p<0.01$) in the captopril plus angiotensin II pretreated group and from 0.2 ± 0.02 to 12.3 ± 4.0 μM ($p<0.05$) in the group receiving no pretreatment. Comparison of the increases observed at 45 minutes revealed no significant difference between the 3 groups.

Figure 6.27 shows serum serotonin concentration in each of the 3 groups throughout the experimental time course. There was no significant difference in basal levels of serotonin between the 3 groups. Following 5HTP administration, serotonin increased maximally at 45 minutes from 2.0 ± 0.6 to 8.3 ± 1.3 μM ($p<0.05$) in the captopril pretreated group, from 1.4 ± 0.4 to 5.7 ± 1.1 μM ($p<0.05$) in the captopril plus angiotensin II pretreated group and from 1.0 ± 0.1 to 6.0 ± 1.4 μM ($p<0.05$) in the group receiving no pretreatment. Comparison of the increases observed at 45 minutes revealed no significant difference between the 3 groups.

Figure 6.28 shows serum 5HIAA concentration in each of the 3 groups throughout the experimental time course. There was no significant difference in basal levels of 5HIAA between the 3 groups. Following 5HTP administration, 5HIAA increased maximally at 45 minutes from 0.9 ± 0.2 to 23.3 ± 8.0 μM ($p<0.05$) in the captopril pretreated group, from 0.6 ± 0.2 to 18.7 ± 3.3 μM ($p<0.05$) in the captopril plus angiotensin II pretreated group and from 0.4 ± 0.1 to 19.0 ± 8.8 μM ($p<0.05$) in the group receiving no pretreatment. Comparison of the increases observed at 45 minutes revealed no significant difference between the 3 groups.

Figure 6.29 shows PRA in each of the 3 groups throughout the experimental time course. Captopril pretreatment alone caused a significant increase in basal PRA, which was 16.1 ± 2.8 compared with 3.7 ±0.7 ng/ml/hr ($p<0.01$) in the untreated group. Captopril plus

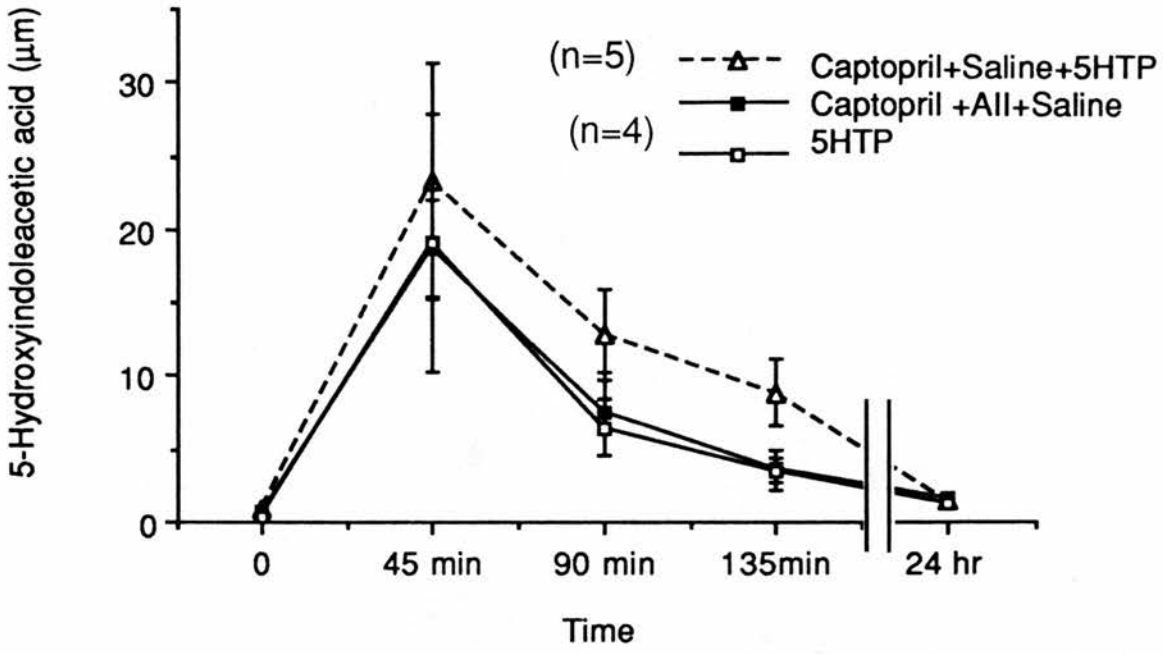


Figure 6.28. **The effect of 5HTP injection on serum 5HIAA concentration in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

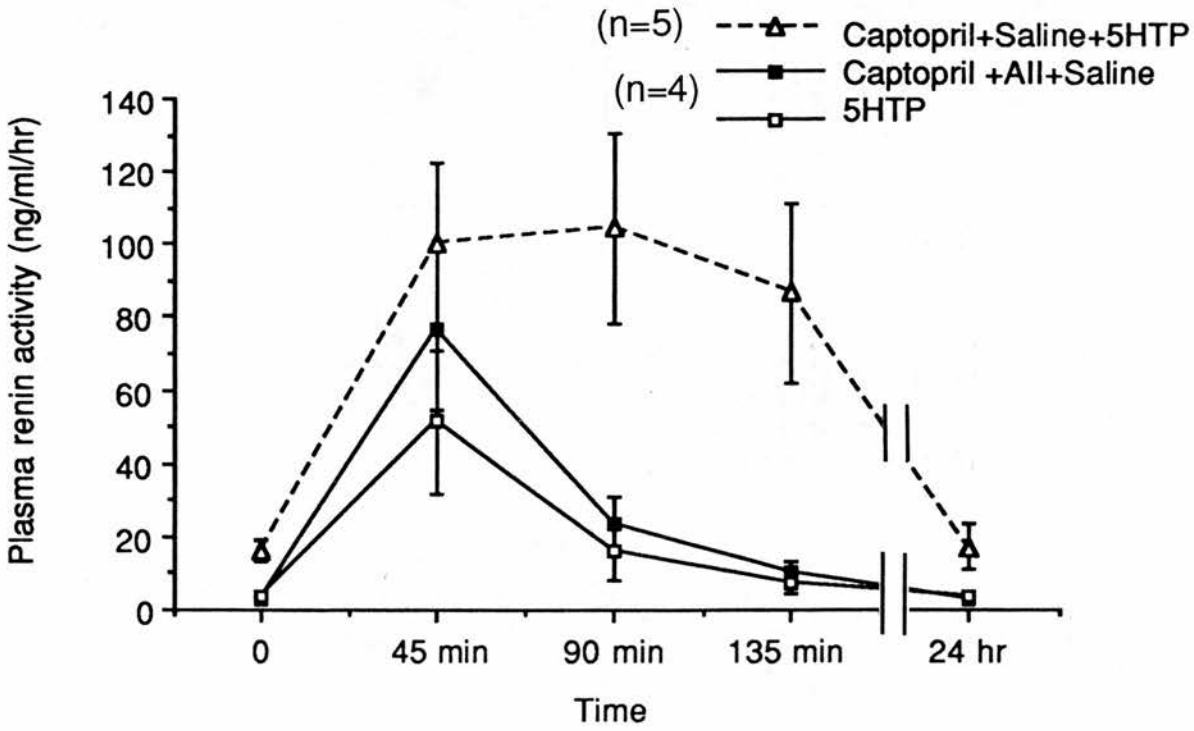


Figure 6.29. **The effect of 5HTP injection on PRA in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

angiotensin II pretreatment did not significantly affect basal PRA which was 3.1 ± 0.7 ng/ml/hr . Following 5HTP administration, PRA increased maximally at 45 minutes from 16.1 ± 2.8 to 100.4 ± 22.2 ng/ml/hr ($p<0.05$) in the captopril pretreated group, from 3.1 ± 0.7 to 76.4 ± 21.8 ng/ml/hr ($p<0.05$) in the captopril plus angiotensin II pretreated group and from 3.7 ± 0.7 to 51.3 ± 19.7 ng/ml/hr ($p<0.05$) in the group receiving no pretreatment. Comparison of the increases observed at 45 minutes revealed no significant difference between the 3 groups. However, the time course of the return to basal levels was considerably prolonged in the captopril only pretreated group.

Figure 6.30 shows plasma corticosterone concentration in each of the 3 groups throughout the experimental time course. There was no significant difference in basal levels of corticosterone between the 3 groups. Following 5HTP administration, corticosterone increased maximally at 45 minutes from 84.4 ± 20.3 to 638.6 ± 80.0 nM ($p<0.001$) in the captopril pretreated group, from 86.8 ± 71.6 to 921 ±158.6 nM ($p<0.05$) in the captopril plus angiotensin II pretreated group and from 91.4 ± 54.2 to 692 ± 130 nM ($p<0.01$) in the group receiving no pretreatment. Comparison of the increases observed at 45 minutes revealed no significant difference between the 3 groups.

Figure 6.31 shows plasma aldosterone concentration in each of the 3 groups throughout the experimental time course. There was no significant difference in basal levels of aldosterone between the 3 groups. Following 5HTP administration, aldosterone increased maximally at 45 minutes from 0.16 ± 0.04 to 2.26 ±0.58 nM ($p<0.05$) in the captopril plus angiotensin II pretreated group, from 0.13 ± 0.01 to 2.38 ± 0.61 nM ($p<0.01$) in the group receiving no pretreatment, and to a lesser degree from 0.17 ± 0.02 to 0.69 ± 0.18 nM ($p<0.05$) in the captopril pretreated group. Comparison of the increase in aldosterone observed at 45 minutes in each of the pretreated groups with the untreated group revealed that captopril pretreatment significantly inhibited the aldosterone response to 5HTP ($p<0.05$).

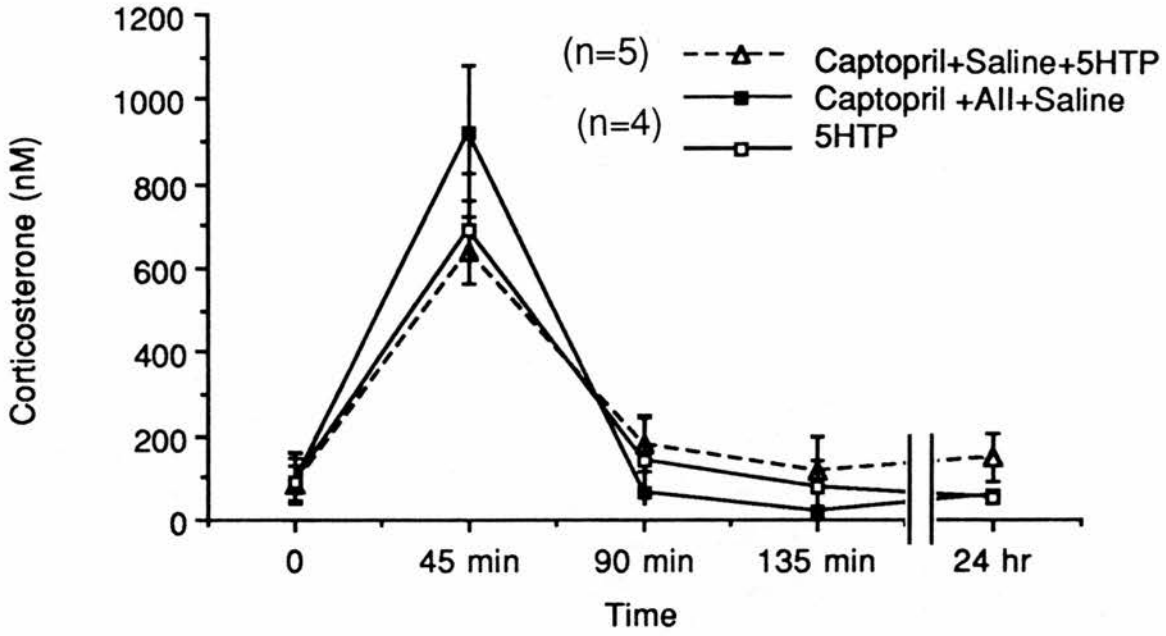


Figure 6.30. **The effect of 5HTP injection on plasma corticosterone concentration in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

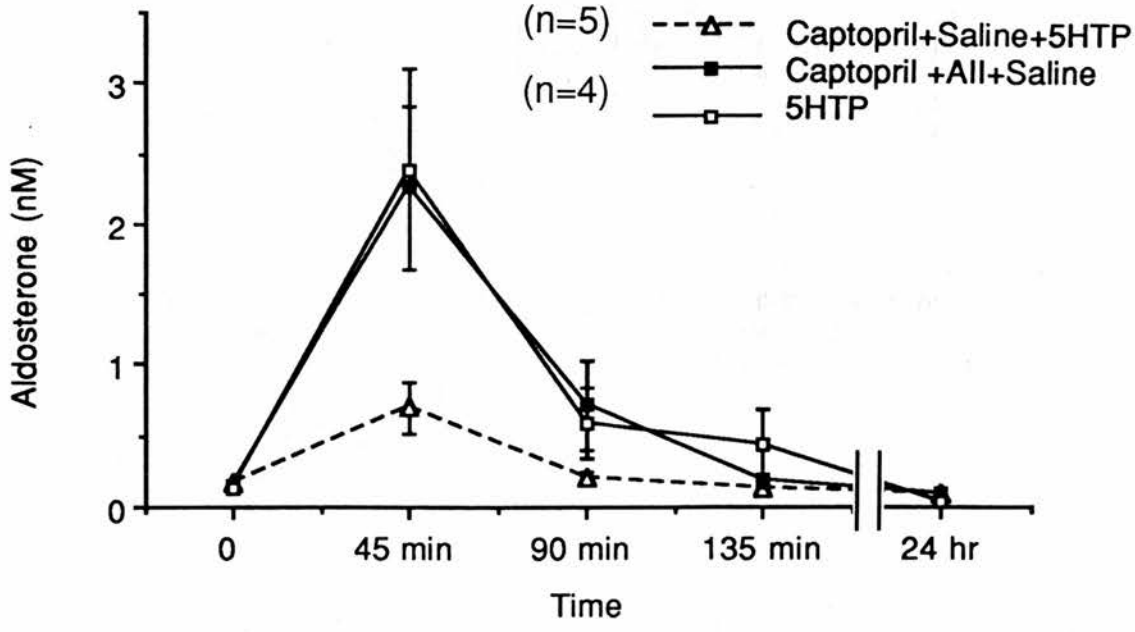


Figure 6.31. **The effect of 5HTP injection on plasma aldosterone concentration in the cannulated rat, untreated or pretreated with captopril or captopril plus angiotensin II.**

However, co-administration of angiotensin II with the captopril returned the response to normal.

Plasma sodium and potassium concentration were not measured in this particular study.

6.6. Discussion

The effect of acute serotonin enhancement *in vivo* on adrenal function, particularly aldosterone secretion, was studied in conscious rats with indwelling arterial cannulae, a technique designed to enable the easy withdrawal of blood without the use of an anaesthetic which would itself lower blood pressure and activate the renin-angiotensin system. Serial blood samples were taken under minimal stress conditions via the cannulae, before and after administration of either saline or 5HTP, the immediate precursor of serotonin. In order to assess the relative involvement of both the renin-angiotensin system and the hypothalamic-pituitary-adrenal axis the study was repeated in animals following pharmacological blockade of one of the systems using captopril and dexamethasone respectively.

5HTP was administered in preference to serotonin itself as it has been reported to show no effect on blood pressure, in contrast to serotonin which decreases blood pressure when peripherally administered to man (Barney *et al* 1981). However, other groups have reported no change in blood pressure when serotonin was administered to man (Mantero *et al* 1982), or a decrease in blood pressure when 5HTP was administered i.p to rats, although the concentrations of 5HTP used were much higher than those utilised in these studies (Henning and Rubenson 1971). A fall in blood pressure would result in stimulation of renin release from the kidney with a consequent activation of the renin-angiotensin system. Administration of 5HTP also allows a slower more controlled increase in the circulating levels of serotonin, which may be rapidly metabolised or taken up into storage sites such as the gut if given directly. However 5HTP, unlike serotonin, crosses the blood brain barrier and may

therefore elicit centrally mediated actions.

In the preliminary study 2 groups of animals were utilised. One group received saline after the collection of the basal blood sample, whilst the other group received 5HTP. The results show that 5HTP administration greatly increased circulating levels of 5HTP itself, 5HIAA and to a lesser degree serotonin, each of which returned to basal levels by the end of the study. Some groups have failed to measure any increase in circulating serotonin following 5HTP administration (Shenker *et al* 1985a, 1985b). Although this study has shown an increase in serotonin levels, the apparent inconsistency between the magnitude of the increase in serotonin compared to that of its precursor and metabolite may be due to a number of reasons. Firstly, serotonin is rapidly metabolised to 5HIAA. Secondly, it may be taken up into extra-circulatory storage sites such as the gut or the adrenal gland, and this would remain undetected in serum estimations. Thirdly, the conversion of 5HTP to serotonin is known to occur both in CNS and in the kidney and this would also remain undetected in serum measurements. Finally, serum levels of 5HTP and 5HIAA are low, whereas plasma serotonin levels are high due to the fact that platelets store serotonin. Therefore, the increase in serotonin is being measured against high endogenous serotonin, whilst the increase in 5HTP and 5HIAA are measured against low plasma levels. However, despite the inconsistency between the serotonin and the 5HTP and 5HIAA responses it must be assumed that at some point the circulating and/or central levels of serotonin are increased to a similar extent as 5HTP and 5HIAA, as this is the only pathway available for conversion of 5HTP to 5HIAA.

In addition to the changes observed in 5HTP, serotonin and 5HIAA concentration , significant increases in PRA , corticosterone and aldosterone were also observed following 5HTP administration. Other groups have reported similar increases in aldosterone following administration of 5HTP, 5HT or tryptophan, although conflicting results surround the increase in PRA and ACTH (Modlinger *et al* 1979, Zimmerman and Ganong 1980, Maestri *et al* 1988, Mantero *et al* 1982, Shenker *et al* 1985a, 1985b). It is unclear at this stage if the increase in

corticosterone results from direct activation of 11-β-hydroxylase enzyme activity or indirectly by a central effect of serotonin on ACTH release from the pituitary. No change in potassium or sodium levels, which may account for the increase in aldosterone secretion, were observed in this or the aforementioned studies by other groups. It is interesting to note that the basal plasma potassium concentration is higher than the accepted physiological concentration of 3.9 mM. There is no increase in plasma sodium concentration, even although aldosterone secretion is enhanced. This is to be expected as it is unlikely that the time course followed would show any changes in plasma sodium as the renal effects of aldosterone commence 1 to 2 hours after steroid secretion, reflecting the time required for protein synthesis. In addition it is more likely that any changes in sodium would be observed in the urine rather than in the plasma.

The control group of animals which received saline showed no significant change in any parameter, indicating that any stress induced by the injection or animal handling was not responsible for the results in the 5HTP treated group.

The preliminary data indicate that acute 5HTP administration stimulates aldosterone secretion *in vivo*. However, it is unclear what the mechanism of this effect is, although the results suggest a number of feasible possibilities. Firstly, serotonin formed from 5HTP may cause changes in blood pressure, as it has been previously been reported. A fall in blood pressure would activate the renin-angiotensin system and manifest itself in the changes in PRA and aldosterone observed in the preliminary study.

Secondly, 5HTP may be converted to serotonin either centrally, peripherally or indeed locally, and act directly on the zona glomeruosa to increase aldosterone and corticosterone secretion, as has previously been reported *in vitro* in isolated cells and in the whole isolated perfused adrenal gland (Müller and Zeigler 1968, Haning *et al* 1970, Bing and Schulster 1977, Hinson and Vinson 1989). The presence of specific receptors for serotonin in the zona glomerulosa has already been discussed in some detail in chapter 4 and has also been reported by a number of other groups. It is known that capsular tissue can metabolise

serotonin to 5HIAA and another unidentified metabolite (Trost and Müller 1976), and that L-aromatic amino acid decarboxylase, the enzyme which converts 5HTP to serotonin, may also be present in the adrenal gland, although localisation within the zona glomerulosa has yet to be investigated (Waugh, Dockrell and Gow, unpublished observation). 5HTP has been shown to have a small stimulatory effect on aldosterone secretion *in vitro*, although the concentration required to evoke a similar response is approximately 100 times greater than serotonin. Presumably this effect is mediated by the action of serotonin which is synthesised from 5HTP by the zona glomerulosa. Further experimentation is required in order to confirm this possibility.

Thirdly, the increase in PRA suggests serotonin may activate the renin-angiotensin system. Renin release from the juxta-glomerular cells of the kidney is controlled by β–adrenergic receptors, renal perfusion pressure and volume, sodium absorption across the macula densa, circulating levels of vasopressin and angiotensin II and the CNS, perhaps by serotoninergic neurones (Reid *et al* 1978, Brosnihan *et al* 1981, Van de Kar *et al* 1981,1982, Zimmerman and Ganong 1980). Serotonin may also increase renin release by lowering blood pressure. Previous studies have given conflicting reports of the effects of serotonin or 5HTP on blood pressure (Henning and Rubenson 1971, Barney *et al* 1981, Mantero *et al* 1982). In order to resolve the question of an indirect effect of 5HTP on blood pressure, the blood pressure of the animals was measured at time = 0, prior to, and for 45 minutes following administration of saline or 5HTP. No significant decrease in blood pressure was observed with either saline or 5HTP which would account for the activation of the renin-angiotensin system. Therefore changes in hormone status secondary to a decrease in blood pressure can be excluded and so renin release mediated by a fall in blood pressure after 5HTP administration seems unlikely. There is however a significant increase in blood pressure within 25 minutes, in the group of animals receiving 5HTP, which is probably secondary to activation of the renin-angiotensin system.

The fourth hypothesis is that serotonin formed within the CNS may act on the

hypothalamus to release CRF, which subsequently acts on the pituitary, increasing ACTH secretion. ACTH would increase both aldosterone and corticosterone secretion from the adrenal cortex. This theory is supported by the large increase in corticosterone secretion observed in the preliminary study following 5HTP administration and also several *in vitro* studies by other groups which have shown that serotonin stimulates CRF release from the hypothalamus and ACTH release from the pituitary (Weiner and Ganong 1978, Kreiger and Kreiger 1979, Fuller 1981).

Finally, the last explanation is that serotonin acts to increase plasma potassium or decrease plasma sodium concentration, both of which would increase aldosterone secretion, resulting in sodium retention and potassium excretion. However, these parameters were measured throughout the study and they showed no consistent significant change which would account for the observed changes in aldosterone secretion.

The involvement of the hypothalamo-pituitary adrenal axis was investigated by conducting an experiment, similar to the preliminary study, with the exception that some of the animals were pretreated with dexamethasone, a synthetic glucocorticoid which inhibits ACTH release from the pituitary gland. Successful dexamethasone suppression was monitored by the decrease in basal corticosterone concentration in the two groups which received it, compared to the control group which received saline. The results show that dexamethasone pretreatment did not impair the uptake of 5HTP or its subsequent conversion to serotonin and 5HIAA. Similarly the PRA response to 5HTP was unaffected. However, the increases in both corticosterone and aldosterone were significantly inhibited, although the aldosterone response was not completely suppressed. This inhibition of the aldosterone response with dexamethasone suggests that the action of serotonin on adrenal function in this model may be mediated, at least in part, by activation of the hypothamo-pituitary adrenal axis, causing release of ACTH which stimulates aldosterone and corticosterone secretion, or that ACTH is required permissively for the central action of serotonin. Centrally mediated actions of serotonin have been suggested by a number of

groups, although the data reported contrasts somewhat with the results obtained in this study and may indicate that other central pathways in addition to the hypothalamo-pituitary adrenal axis may mediated the action of serotonin. Dexamethasone blocked the increase in ACTH and cortisol observed with 5HTP administration in man, but had no effect on aldosterone (Maestri *et al* 1988, Shenker *et al* 1985a, 1985b). Blockade of the peripheral conversion of 5HTP to serotonin with carbidopa, which allows complete central elevation of serotonin, enhanced the aldosterone response to 5HTP. However, it must be taken into consideration that carbidopa also blocks the formation of dopamine which inhibits aldosterone secretion. Therefore, the enhancement of aldosterone may be due to decreased production of dopamine rather than a centrally mediated action of serotonin. For this reason carbidopa was not utilised in any of these studies.

The effect of serotonin on the renin-angiotensin system was studied in animals pretreated with the ACE inhibitor captopril, which prevents the formation of angiotensin II from angiotensin I. Successsful blockade of the renin-angiotensin system was judged by the increase in PRA in the captopril treated groups. This is due to the loss of the negative feedback system, whereby increases in angiotensin II act to suppress renin secretion and the subsequent formation of angiotensin II. The results show that captopril pretreatment did not impair the uptake of 5HTP or its conversion to serotonin and 5HIAA. Similarly there was no effect on the corticosterone respose. The PRA response was also unaffected, although at first sight it seems the reponse in the captopril pretreated group was enhanced, however basal PRA was much higher in this group. The time required to return to basal PRA following 5HTP administration was prolonged in the group receiving captopril. This could be due to the blockade of angiotensin II formation which cannot act to suppress further renin release from the kidney, as it does in the untreated group. The aldosterone response to 5HTP was inhibited in the presence of captopril, though not completely.

The curtailment of the aldosterone response with ACE inhibition suggests that serotonin increases PRA and subsequently angiotensin II levels, which act directly at the zona

glomerulosa to increase aldosterone secretion. Activation of the renin-angiotensin system by serotonin and its precusors has been shown by many other groups. Peripheral administration of 5HTP in rats results in a potent dipsogenic effect accompanied by an increase in PRA . The dipsogenic effect can be blocked with carbidopa, propanolol, captopril and methysergide, suggesting that 5HTP is converted to serotonin peripherally, as carbidopa blocks peripheral formation of serotonin, and then acts on specific serotonin and β-adrenergic receptors, increasing renin release from the kidney. Similar results were observed with serotonin itself, with the exception that carbidopa had no inhibitory effect (Kikta *et al* 1981, 1983, Threatte *et al* 1981). The drinking response to serotonin but not 5HTP could be abolished by bi-lateral nephrectomy, suggesting that thirst induced by serotonin is mediated exclusively by the renal renin-angiotensin system, whereas 5HTP induced drinking may also involve a renin-independent possibly centrally mediated mechanism (Rowland *et al* 1987). Serotoninergic control of renin release was also postulated by Van de Kar *et al* 1981, who showed, conversely, that depletion of serotonin levels using PCPA or 5,7-dihydroxytryptamine decreases PRA, although other groups have shown no effect (Shisheva *et al* 1987, Mikulic *et al* 1988). The mechanism by which activation of serotonin receptors results in renin release from the kidney is not well understood and it was not attributed to a decrease in blood pressure by these groups. However, it may involve the release of catecholamines from the adrenal medulla, or a centrally activated pathway. A central activation of the renin-angiotensin system was postulated by Zimmerman and Ganong 1980, who showed that the stimulatory effect of 5HTP and tryptophan on renin secretion in anaesthetised dogs could be inhibited by renal denervation, blockade of serotonin receptors and inhibition of serotonin formation within the brain.

Although other groups have shown no activation of the renin-angiotensin system with administration of 5HTP or serotonin (Maestri *et al* 1988, Mantero *et al* 1982, Shenker *et al* 1985a, 1985b), the results of this study show a clear increase in PRA. However, it is unclear from the study if aldosterone secretion is increased by the action of angiotensin II on the zona

glomerulosa or that normal circulating levels of angiotensin II are required permissively for the action of serotonin on the adrenal or its action on another system such as the CNS.

Depletion of circulating angiotensin II levels during a sodium replete diet results in the down regulation of adrenal angiotensin II receptors, which decrease in receptor number and binding affinity, and so reduce the aldosterone response to angiotensin II (Glossman *et al* 1974, Aguilera *et al* 1978, Aguilera *et al* 1980). This regulatory effect may cause similar changes in adrenal and / or other serotonin receptors which, from the work of chapter 4 and that of other groups, seems to interact synergistically in some way with the angiotensin II receptor. Indeed it has been shown that sodium depletion enhances the *in vitro* aldosterone response to angiotensin II and serotonin, whilst sodium loading diminishes the response (Aguilera *et al* 1978, Al-Dujaili *et al* 1982). Similar changes in the adrenal are observed with captopril treatment as this effectively decreases circulating levels of angiotensin II. However, with regard to the role of angiotensin II within the CNS, it is unclear if captopril would cross the blood-brain barrier and block the central renin-angiotensin system, and if the effects of ACE inhibition on the angiotensin II receptor observed in the adrenal, extend to peripheral angiotensin and serotonin receptors, as alterations in sodium balance have the opposite effects in the vascular system as they have in the adrenal (Gunther *et al* 1980, Aguilera *et al* 1981b).

The permissive, rather than mediatory, role of angiotensin II in the action of 5HTP was investigated by comparing the effect of 5HTP on captopril pretreated animals inserted with osmotic mini-pumps delivering normal circulatory levels of angiotensin II, but concomitantly blocking activation of the renin-angiotensin system itself. The response to 5HTP in this group was compared with a group receiving captopril alone and another receiving no pretreatment. The aldosterone levels in this particular study were somewhat lower than in the other experiments. This may be due to a small overdose of heparin, which is known to decrease aldosterone secretion (Abbott *et al* 1966).

Successful normalisation of circulating levels of angiotensin II was monitored by basal

PRA which, compared to the untreated group, is elevated in the group receiving captopril, due to the loss of the negative feedback as previously explained, and normal in the group receiving captopril plus angiotensin II, due to the presence of the negative feedback, and inhibition of further renin release by the angiotensin II. Angiotensin II measurements would have provided a clearer picture, however the assay requires a large volume of blood (>5ml) which was not possible to take in these studies.

The results from this experiment clearly demonstrate that blockade of the renin angiotensin system using captopril does not affect the aldosterone response to 5HTP if circulating angiotensin II levels remain normal, suggesting that the increase in PRA and subsequent increase in angiotensin II caused by 5HTP administration does not mediate the increase in aldosterone secretion. They do however suggest that angiotensin II is required permissively for the action of 5HTP on aldosterone secretion. The exact site of this requirement is unclear. As previously discussed it may be at the site of the adrenal gland where it is required to maintain adrenal responsiveness to serotonin or indeed ACTH, as the dexamethasone experiments suggest the aldosterone response is mediated by release of ACTH and it has been shown by other groups that the adrenal responsiveness to ACTH is dependent on angiotensin II (Venning *et al* 1962, Kinson and Singer 1968, Müller and Huber 1969). Another possibility is that normal circulating levels of angiotensin II are required within the CNS for the action of serotonin, indeed some studies have shown that angiotensin II promotes ACTH release from the pituitary gland (Sobel *et al* 1983). However, although a central renin-angiotensin system does clearly exist (Ganten and Speck 1978), it is unclear if peripheral adminisatration of captopril would cross the blood brain barrier and decrease central levels of angiotensin II, although some studies by other groups suggest that systemic administration of captopril does inhibit the brain renin-angiotensin system in rats (Scholkens *et al* 1983, Gepetti *et al* 1987).

Although acute enhancement of serotonin seems to act to increase aldosterone secretion by a largely indirect mechanism, it must be assumed that the activation of these

systems is mediated by serotonin receptors. Studies with serotonin antagonists were not carried out, mainly because of the uncertainty of the class of receptor involved, and also because of the possible effects on other parameters such as blood pressure, which has been shown to decrease following ketanserin administration (Fozard 1982). This would itself activate the renin-angiotensin system and make clear interpretation of the results very difficult. In addition, the use of these antagonists would not distinguish between peripheral and centrally mediated events, as presumably serotonin elicits its action in either system by activating specific receptors.

In summary, the results of this chapter clearly show that administration of 5HTP, which increases circulating levels of serotonin, activates the renin-angiotensin system, the hypothalamo-pituitary axis and aldosterone secretion by the adrenal gland. However, the actual increase in aldosterone secretion does not appear to be mediated by activation of the renin-angiotensin system, although angiotensin II is required in a permissive capacity for steroidogenesis. This exact site of this remains unclear and requires further investigation. A permissive role of ACTH is also equally possible. However, experiments with osmotic pumps delivering ACTH were not conducted because of the inability of the pumps to mimic the diurnal rhythm of ACTH secretion, which in the nocturnal rat reaches a peak in the late afternoon and a nadir in the early morning (Matsuyama *et al* 1971).

The apparent failure of both captopril and dexamethasone to inhibit the aldosterone response to 5HTP completely, suggests that another mechanism is also operative and this may be a direct action of serotonin on the adrenal cortex. Therefore, the full steroidogenic response to 5HTP involves a complex interplay between the CNS, the renin-angiotensin system and perhaps a direct effect of serotonin at the level of the zona glomerulosa. Any alterations in this fine equilibrium may result in altered adrenal responsiveness leading to changes in mineralocorticoid secretion. Further studies using nephrectomised or hypophysectomised animals may be useful in establishing the relative contribution of the renin-angiotensin system or the hypothalamo-pituitary adrenal axis in the stimulation of

aldosterone secretion by serotonin *in vivo*.

Chapter Seven

Serotoninergic Control of Adrenal Growth.

7.1. Introduction

It is well documented that altered sodium status results in changes in both the width and the responsiveness of the zona glomerulosa, in that a sodium deplete diet increases the width and the responsiveness to angiotensin II, whilst a sodium replete diet has the opposite effect. These effects are thought to be mediated respectively by an up-regulation and down-regulation of the angiotensin II receptor.

Although serotonin increases aldosterone secretion both *in vivo* and *in vitro*, its importance as a trophic factor for the zona glomerulosa has until now received little attention. The possibility that serotonin may act as a trophic factor was studied in rats in which serotonin status was enhanced pharmacologically by administration of 5HTP.

7.2. Aims of study

1. To assess the role of serotonin as a trophic factor by measuring zona glomerulosa and zona fasciculata / reticularis width following chronic administration of 5HTP, the immediate precursor of serotonin.
2. To compare any changes in zona glomerulosa width observed with 5HTP with those observed during altered sodium status.
3. To assess the contribution of the renin-angiotensin system in the effects caused by 5HTP administration by measuring PRA, angiotensin II and also zona glomerulosa width following administration of 5HTP and 5HTP plus the ACE inhibitor captopril.
4. To assess the contribution of the hypothamo-pituitary adrenal axis in the effects caused by 5HTP by measuring corticosterone and zona glomerulosa width following administration of 5HTP and 5HTP plus the synthetic glucocorticoid dexamethasone.

7.3. Materials and methods

The materials and methods for this chapter have been fully described in chapter 3. Briefly, animals were maintained on various dietary regimes for periods of up to 2 weeks. The

animals were then sacrificed and a blood sample taken for estimation of angiotensin II, PRA, aldosterone, corticosterone and serotonin by RIA. The adrenal glands were removed, snap frozen, sectioned, stained and the zona glomerulosa (ZG) and zona fasciculata / reticularis (ZFR) width measured. The staining method utilised haematoxylin and oil red-O, which are nuclear and lipid stains respectively. Using this method the capsule and zona glomerulosa stained dark blue, the zona fasciculata / reticularis stained red and the medulla stained light blue. The zona fasciculata and zona reticularis were measured only in the preliminary studies and together as it was technically difficult to distinguish accurately between the two zones.

7.4. Statistical analysis

All the results are illustrated as mean ± SEM. Statistical significance was calculated using Student's t-test for unpaired samples. A p value of < 0.05 was considered significant. NS indicates non-significance. The p values obtained are quoted in the text and are illustrated on the figures. *, ** and *** on the figures indicates that $p<0.05$, $p<0.01$ and $p<0.001$ respectively.

7.5. Results

7.5.1. The effect of 5HTP administration on serum serotonin concentration

A preliminary study was carried out to assess whether chronic administration of 5HTP in the animals drinking water increased the circulating levels of serotonin. The 5HTP dietary regime is given in chapter three section 3.8.1.

Figure 7.1 shows the circulating levels of serotonin in normal animals (controls) and in those receiving 5HTP in their drinking water for 1 and 2 weeks. Serum serotonin increased from 4.7 ± 0.8 μM in the control group to 14.5 ± 1.9 μM ($p<0.001$) and 10.4 ± 1.1 μM ($p<0.001$) after 1 and 2 weeks of 5HTP treatment respectively.

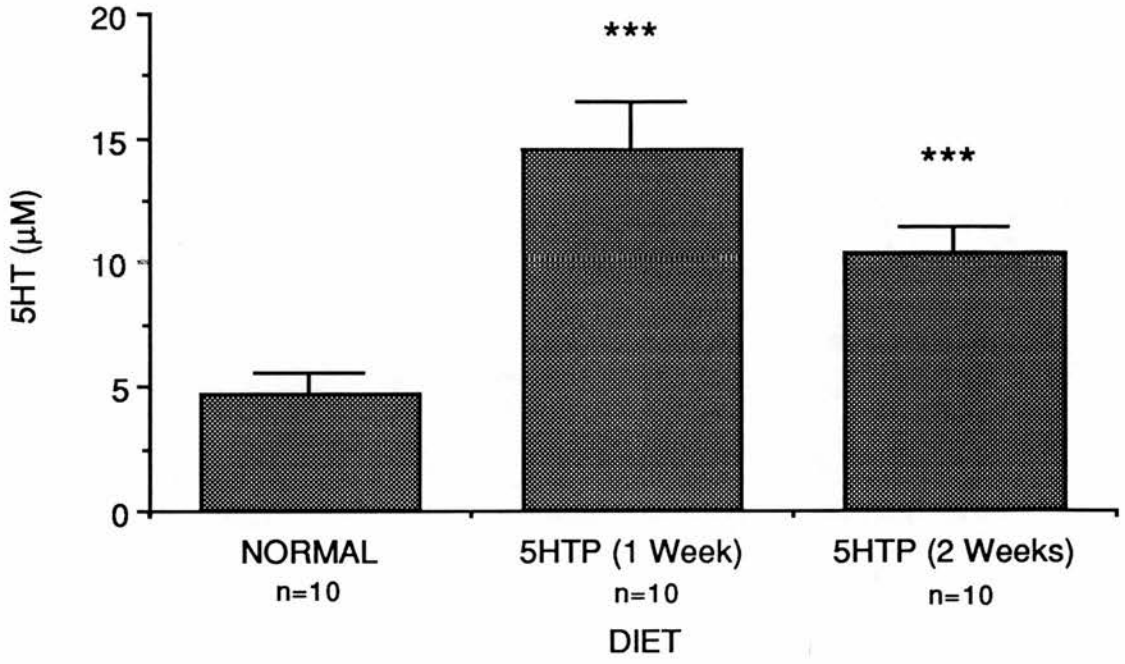
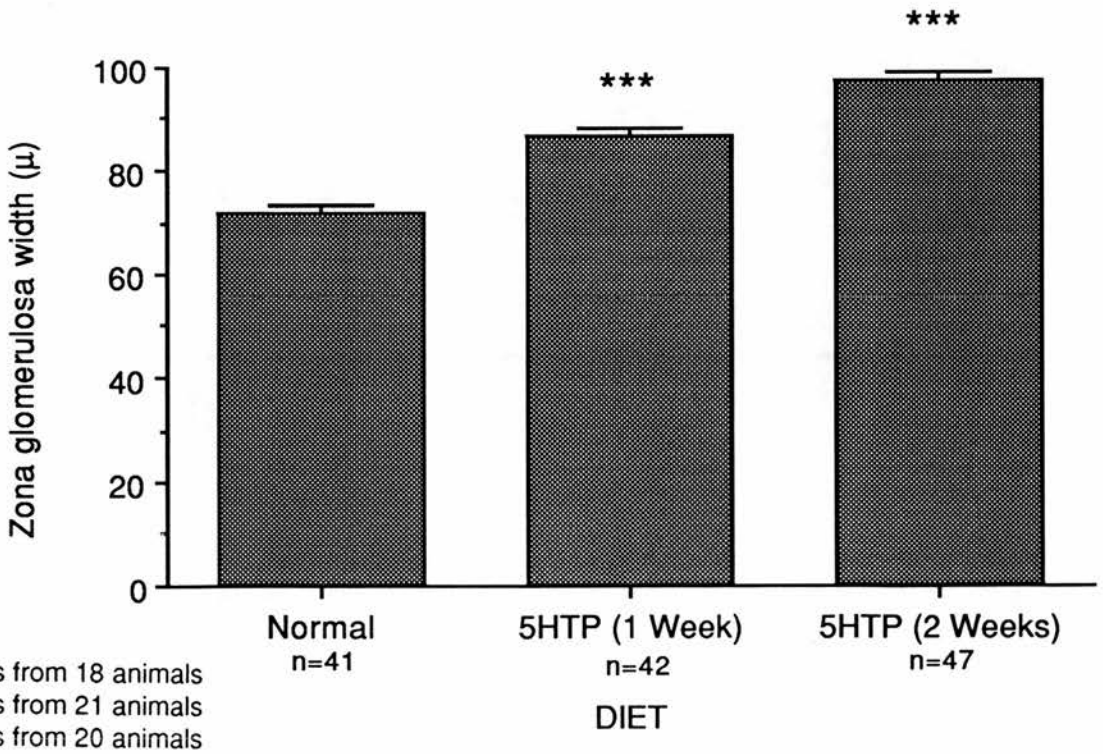


Figure 7.1. **The effect of 5HTP administration on serum serotonin concentration.**



41 sections from 18 animals
42 sections from 21 animals
47 sections from 20 animals

Figure 7.2. **The effect of 5HTP administration on zona glomerulosa width.**

7.5.2. The effect of 5HTP administration on zona glomerulosa and zona fasciculata / reticularis width

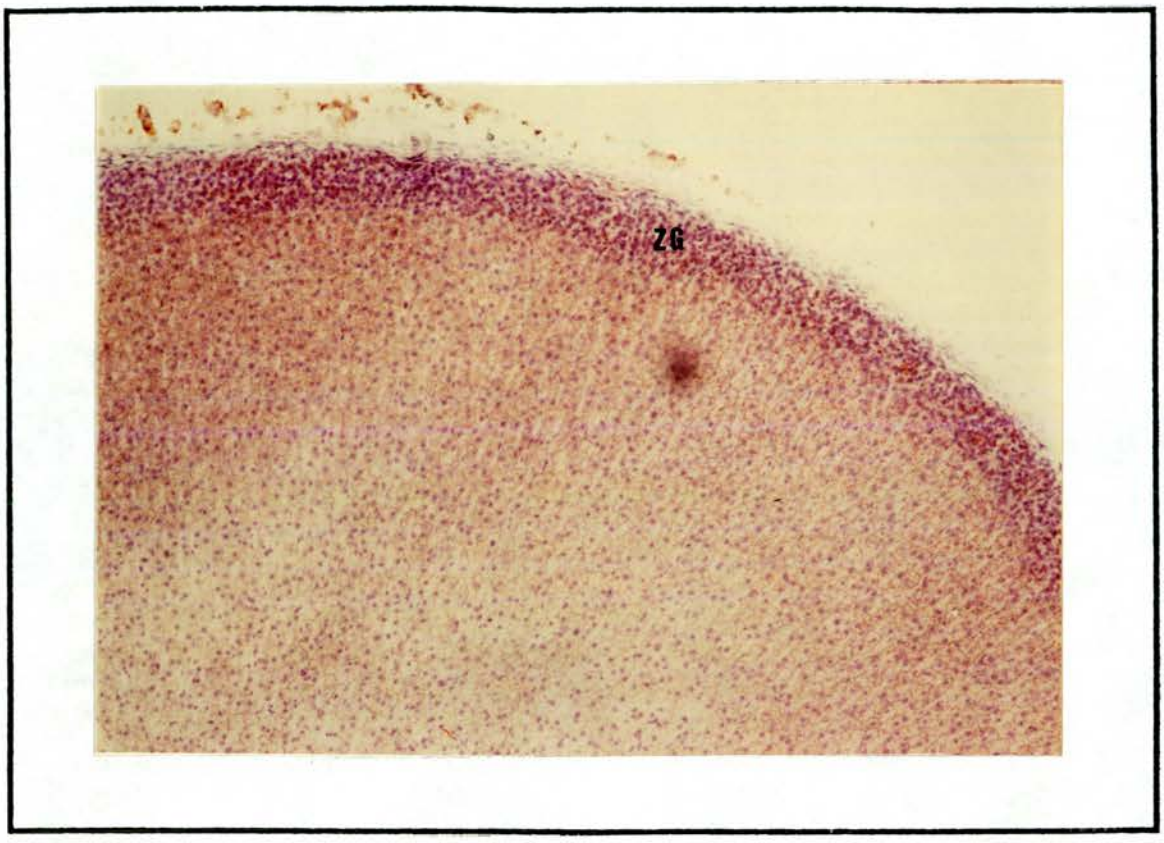
Having established that 5HTP administation, via the animals drinking water, does increase the circulating concentration of serotonin, the effect of this diet on the width of the zona glomerulosa and zona fasciculata / reticularis was studied.

Figure 7.2 shows the width of the zona glomerulosa after 1 and 2 weeks of 5HTP treatment. The width of the zone in the control animals showed no significant change between weeks 1 and 2, therefore the values from each week were pooled. The zona glomerulosa width increased from 72 $\pm$ 1.4 μ in the control group to 86.8 $\pm$ 1.4 μ ($p<0.001$) and 97.6 $\pm$ 1.4 μ ($p<0.001$) after 1 and 2 weeks of 5HTP treatment respectively. Representative photographs of adrenal sections (zona glomerulosa) under normal conditions and after 1 and 2 weeks of 5HTP treatment are illustrated in figures 7.3, 7.4 and 7.5.

Figure 7.6 shows the width of the zona fasciculata / reticularis after 1 and 2 weeks of 5HTP treatment. The width of the zone in the control animals showed no significant change between weeks 1 and 2, therefore the values from each week were pooled. The zona fasciculata / reticularis width decreased from 1216 $\pm$16 μ in the control group to 1085 $\pm$ 23 μ ($p<0.001$) and 980 $\pm$ 30 μ ($p<0.001$) after 1 and 2 weeks of 5HTP treatment respectively.

7.5.3. The effect of sodium depletion and sodium loading on zona glomerulosa and zona fasciculata / reticularis width

It seems clear from the previous section that chronic administration of 5HTP has a trophic effect on the zona glomerulosa and a parallel atrophic effect on the zona fasciculata / reticularis. In order to assess the magnitude of this response and provide a control for the methodology, the effect of altering sodium status on the width of the zones was also studied. The sodium diets used are given in chapter 3 section 3.8.1.



100 microns

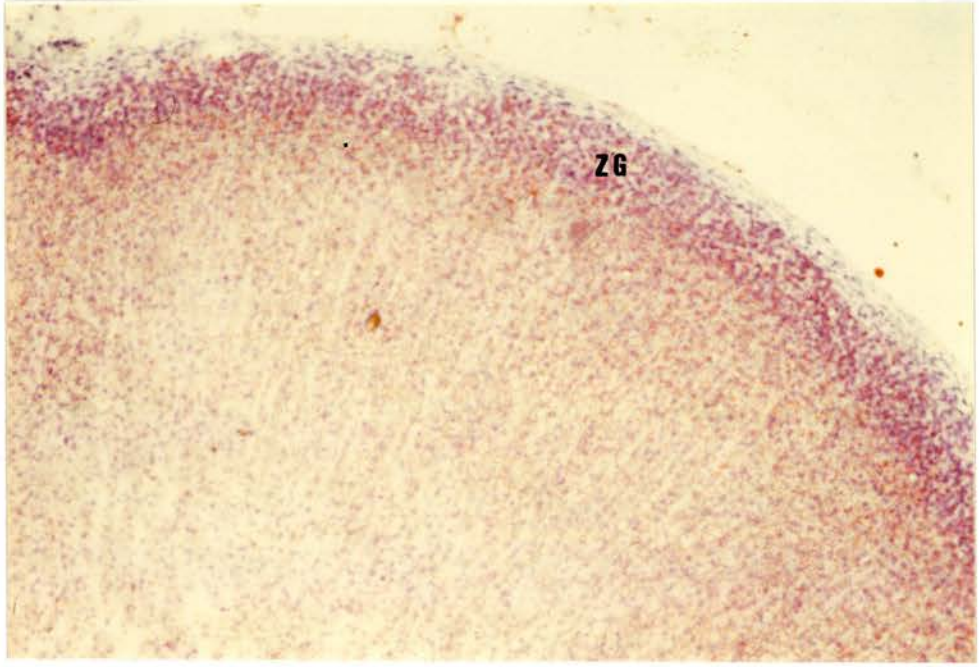
Figure 7.3.

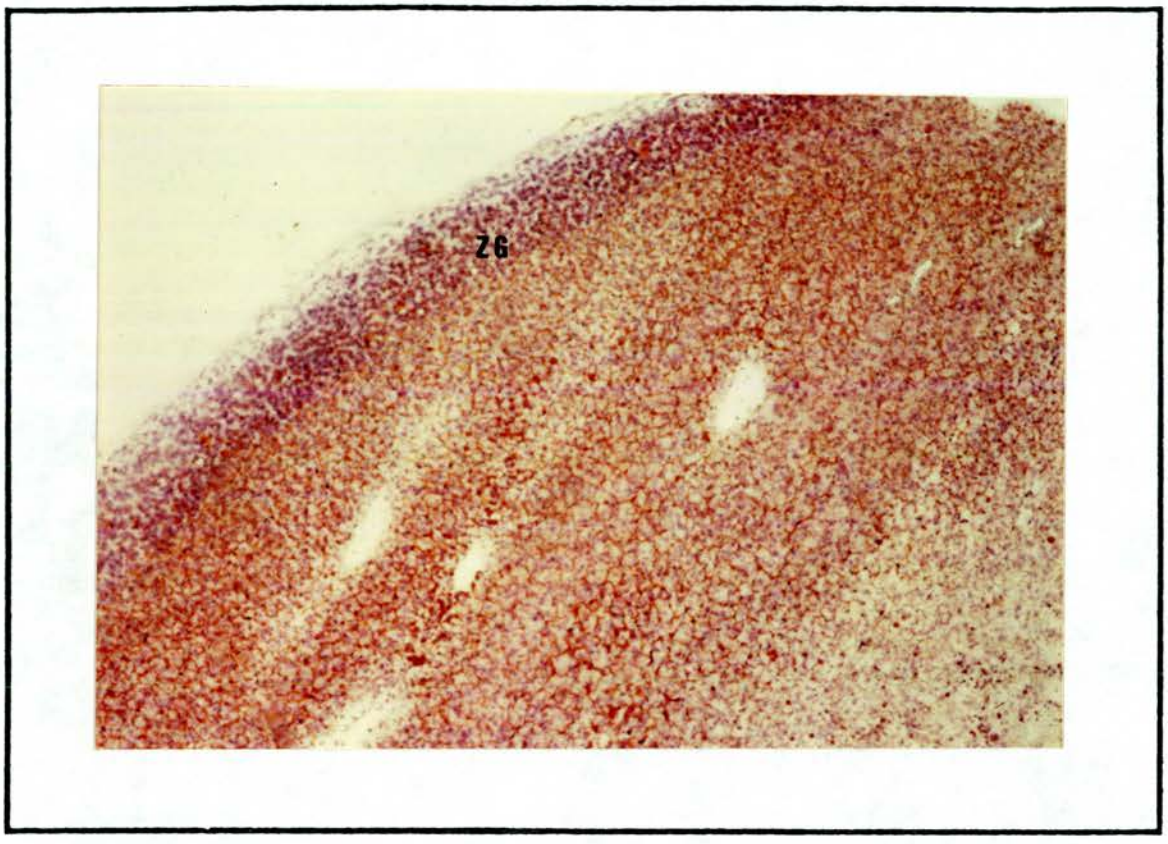
A representative photograph of the adrenal zona glomerulosa under normal conditions.

Figure 7.4.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of 5HTP treatment.

100 microns





100 microns

Figure 7.5.

A representative photograph of the changes in zona glomerulosa width observed after 2 weeks of 5HTP treatment.

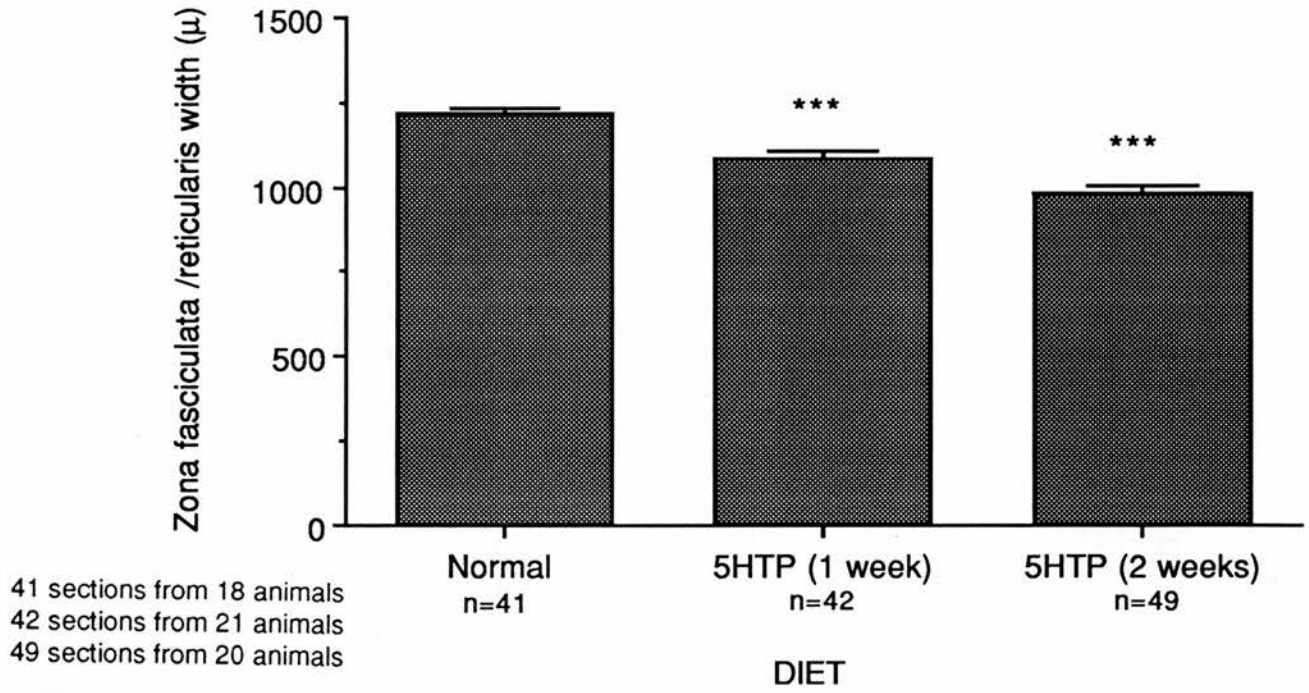


Figure 7.6. **The effect of 5HTP administration on zona fasciculata / reticularis width.**

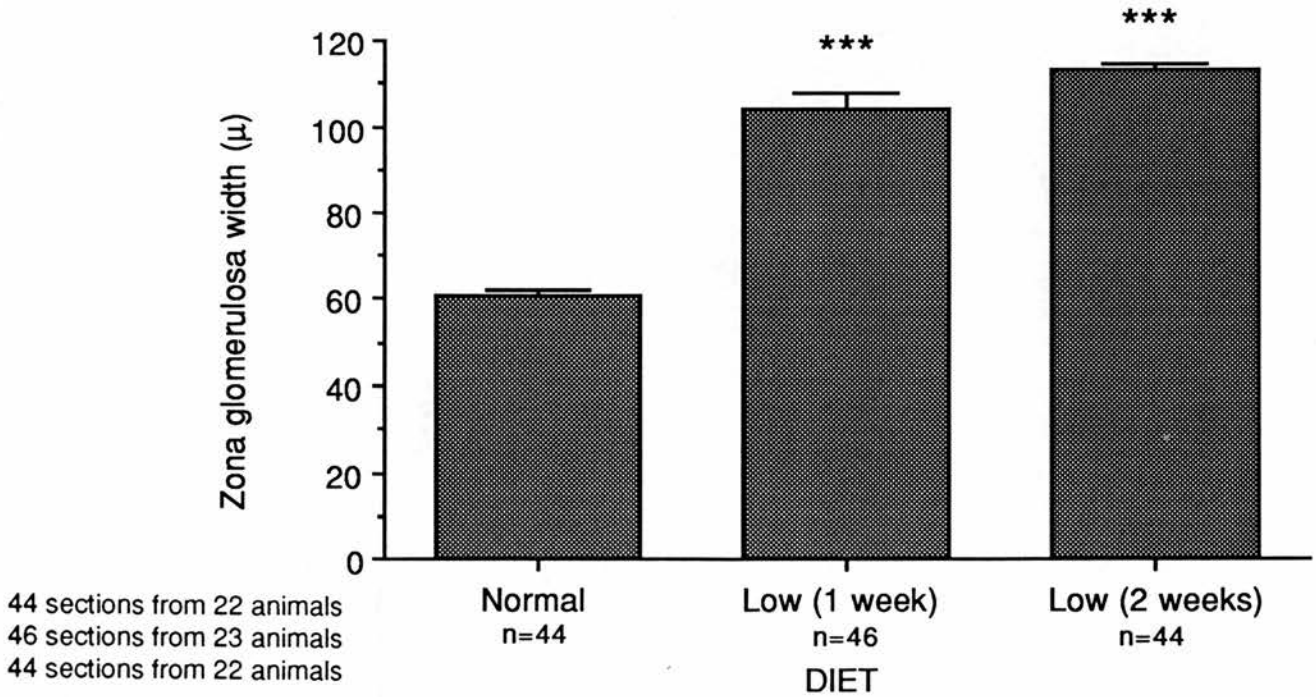


Figure 7.7. **The effect of sodium depletion on zona glomerulosa width.**

Figure 7.7 shows the width of the zona glomerulosa after 1 and 2 weeks of sodium depletion. The width of the zone in the control animals showed no significant change between weeks 1 and 2, therefore the values from each week were pooled. The zona glomerulosa width increased from 60.8 ± 1.2 μ in the control group to 104 ± 3.4 μ ($p<0.001$) and 113.4 ± 1.4 ($p<0.001$) after 1 and 2 weeks of sodium depletion respectively. Representative photographs of adrenal sections (zona glomerulosa) after 1 and 2 weeks of sodium depletion are illustrated in figures 7.8 and 7.9.

Figure 7.10 shows the width of the zona fasciculata / reticularis after 1 and 2 weeks of sodium depletion. The width of the zone in the control animals showed no significant change between weeks 1 and 2, therefore the values from each week were pooled. The zona fasciculata / reticularis width in the control group was 1182 ± 20 μ compared to 1209 ± 19 μ (NS) and 1027 ±16 μ ($p<0.001$) after 1 and 2 weeks of sodium depletion respectively.

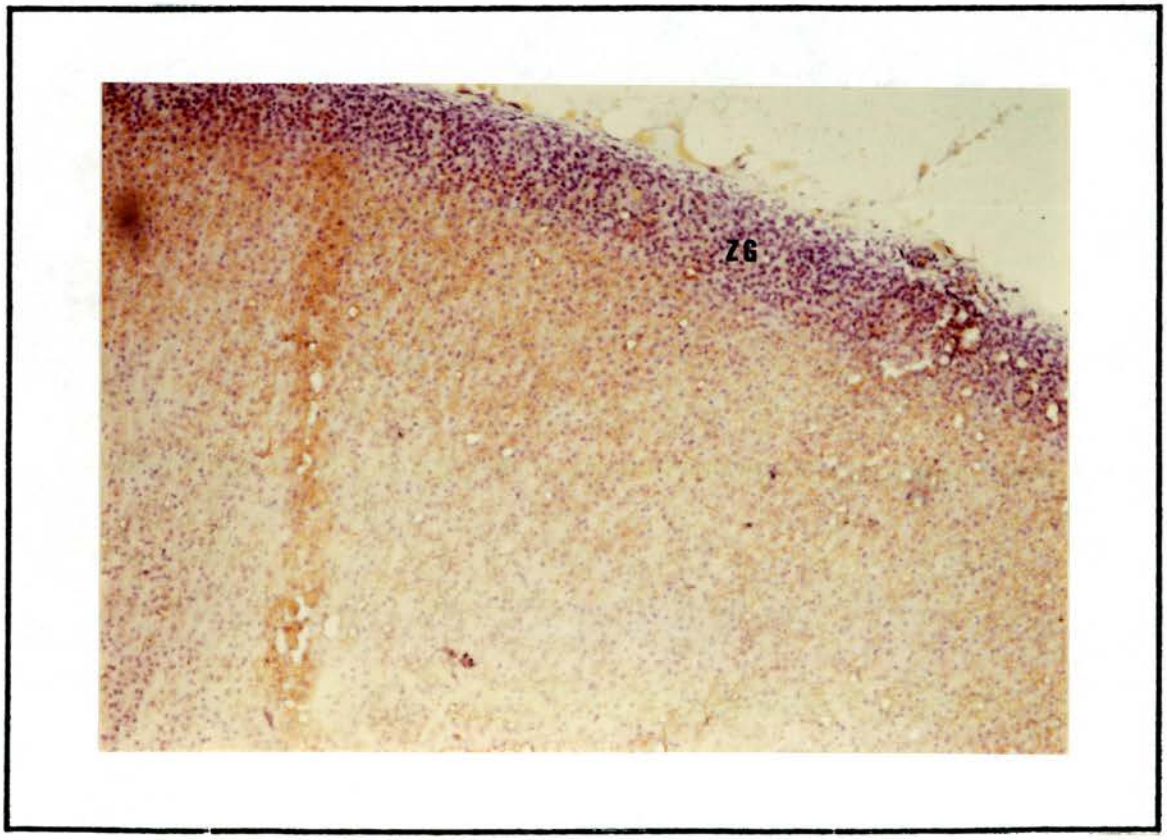
Figure 7.11 shows the width of the zona glomerulosa after 1 and 2 weeks of sodium loading. As this was carried out at the same time as the previous sodium depletion experiment, the same normal values are used. Sodium loading caused the zona glomerulosa width to decrease from 60.8 ± 1.2 μ in the normal group to 55.6 ± 1.3 μ ($p<0.01$) and 57.2 ± 0.6 μ ($p<0.05$) after 1 and 2 weeks of treatment respectively. Representative photographs of adrenal sections (zona glomerulosa) after 1 and 2 weeks of sodium loading are illustrated in figures 7.12 and 7.13.

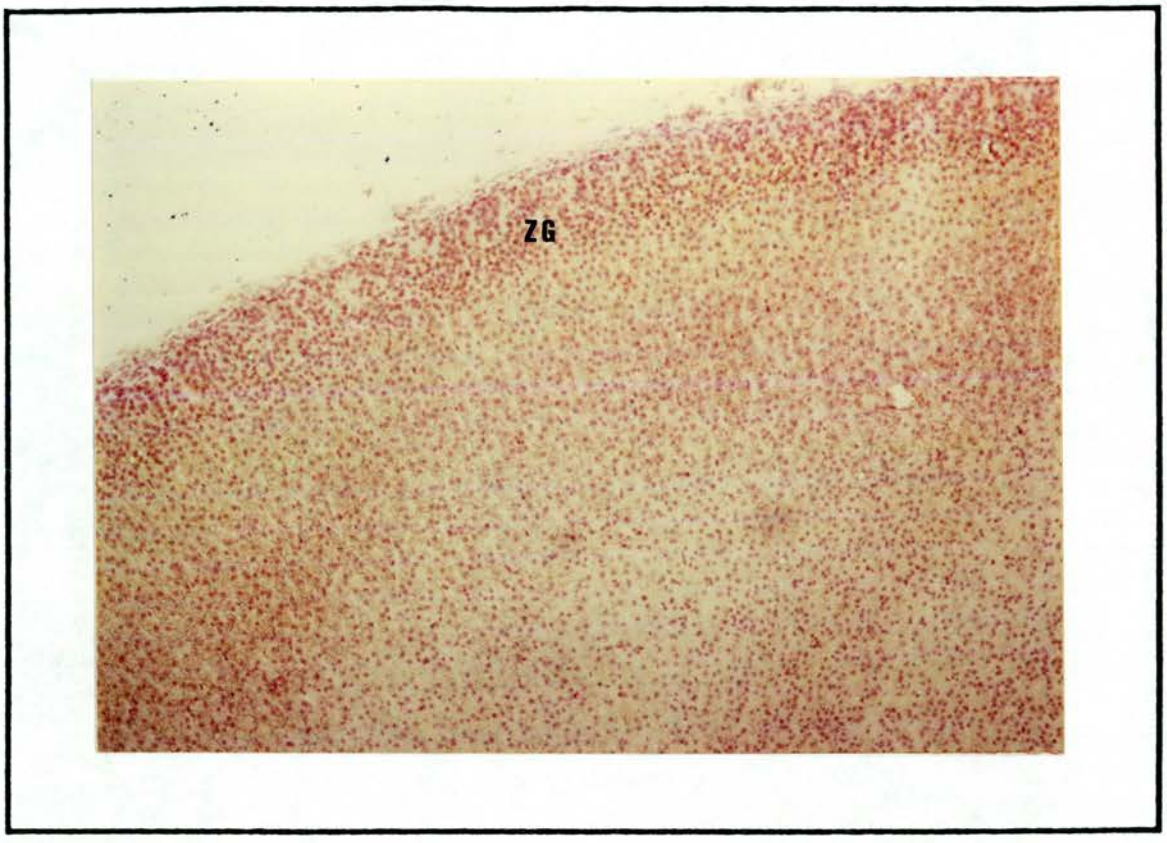
Figure 7.14 shows the width of the zona fasciculata / reticularis after 1 and 2 weeks of sodium loading. The width of the zone in the control animals showed no significant change between weeks 1 and 2, therefore the values from each week were pooled. The zona fasciculata / reticularis width in the control group was 1182 ± 20 μ, compared to 1099 ± 30 μ ($p<0.05$) and 1165 ± 15 μ (NS) after 1 and 2 weeks of sodium loading respectively.

Figure 7.8.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of sodium depletion.

100 microns





100 microns

Figure 7.9.

A representative photograph of the changes in zona glomerulosa width observed after 2 weeks of sodium depletion.

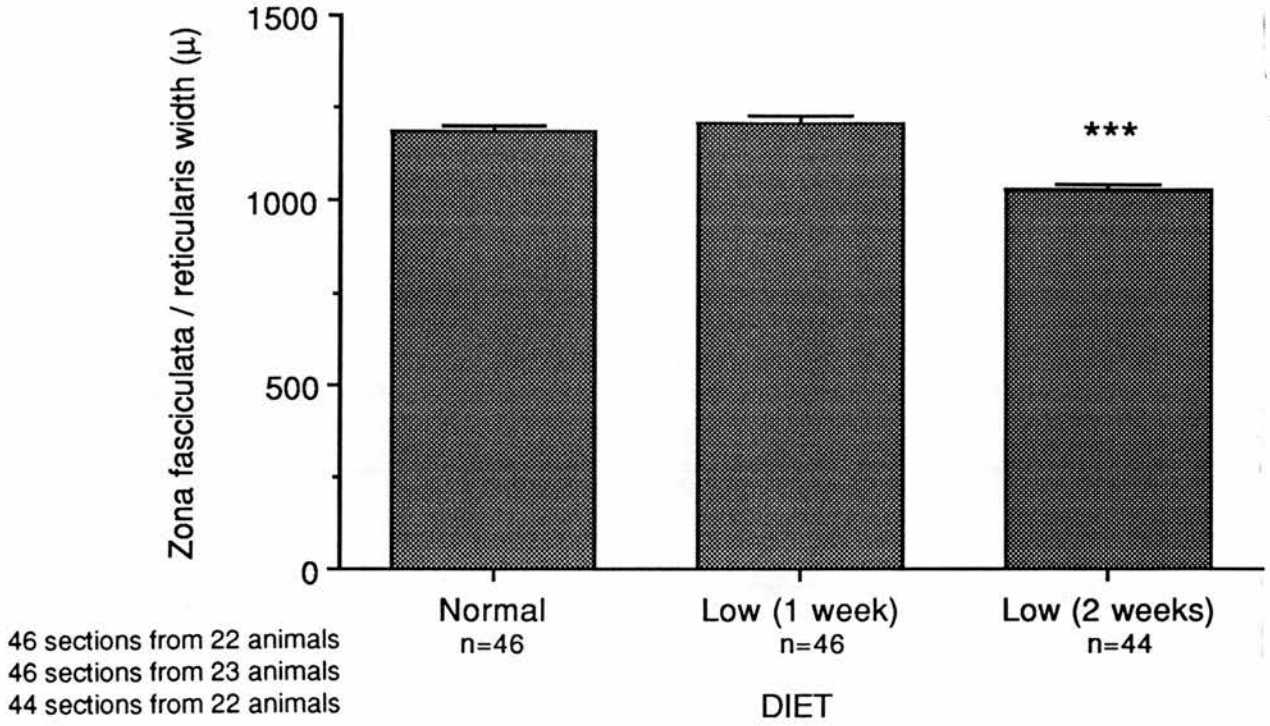


Figure 7.10. **The effect of sodium depletion on zona fasciculata / reticularis width.**

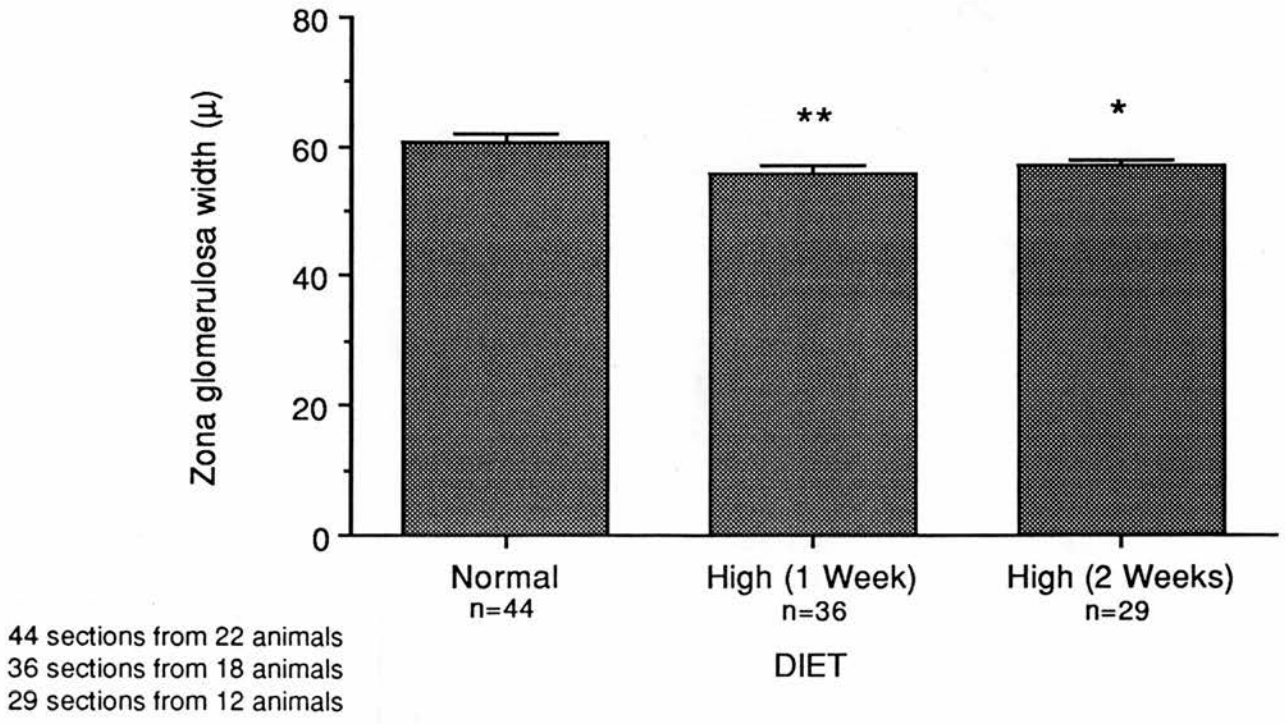
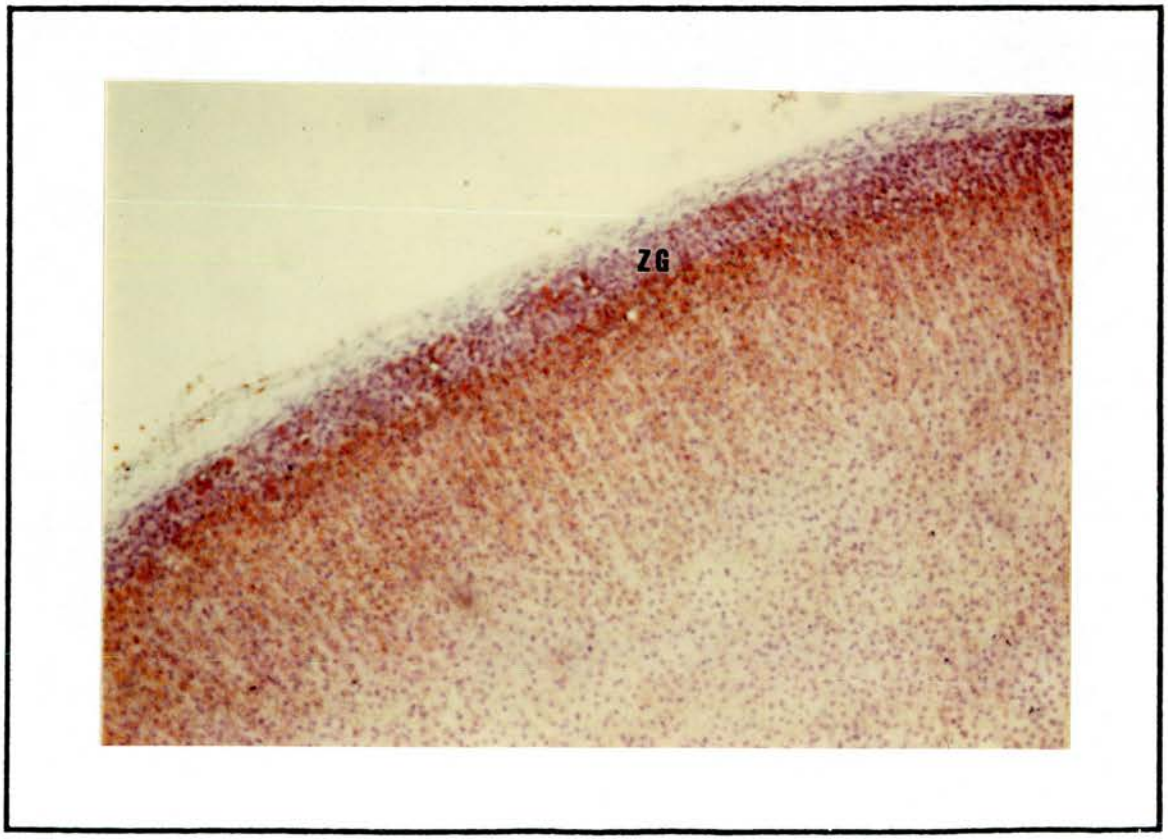


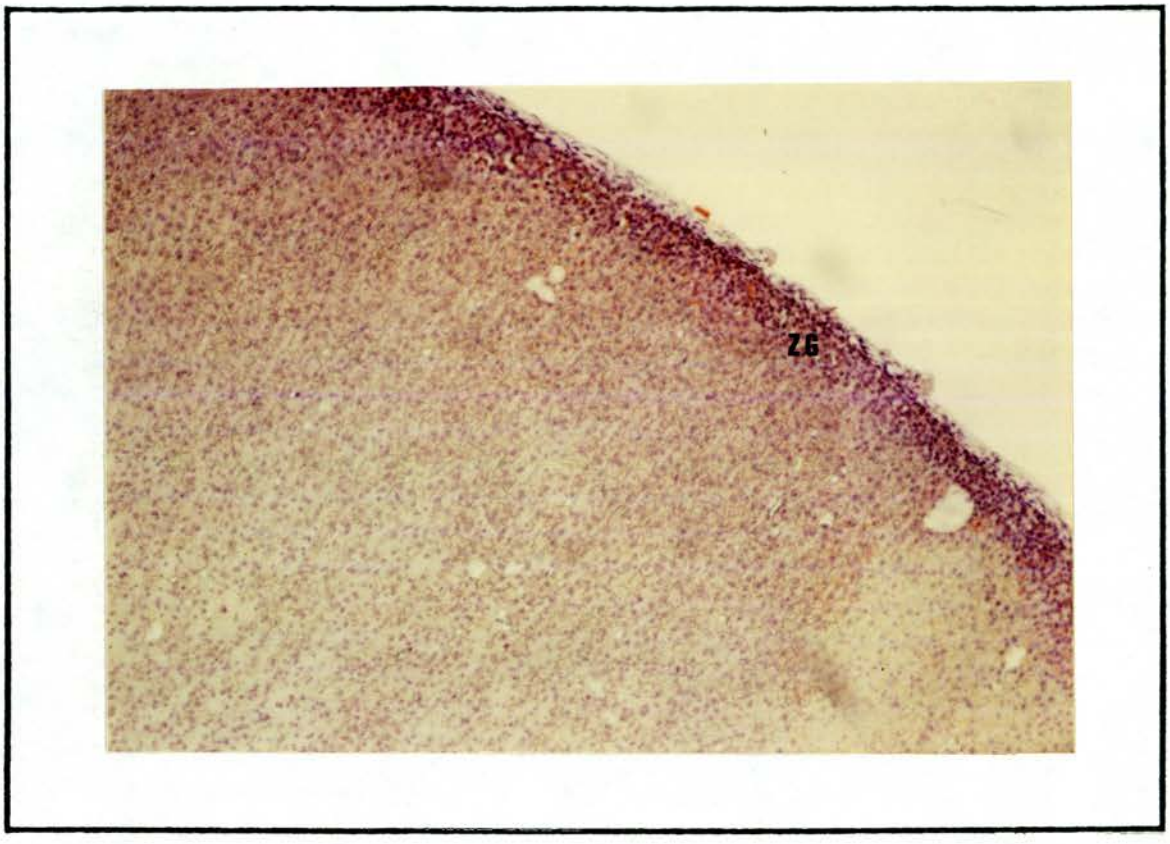
Figure 7.11. **The effect of sodium loading on zona glomerulosa width.**

Figure 7.12.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of sodium loading.

100 microns





100 microns

Figure 7.13.

A representative photograph of the changes in zona glomerulosa width observed after 2 weeks of sodium loading.

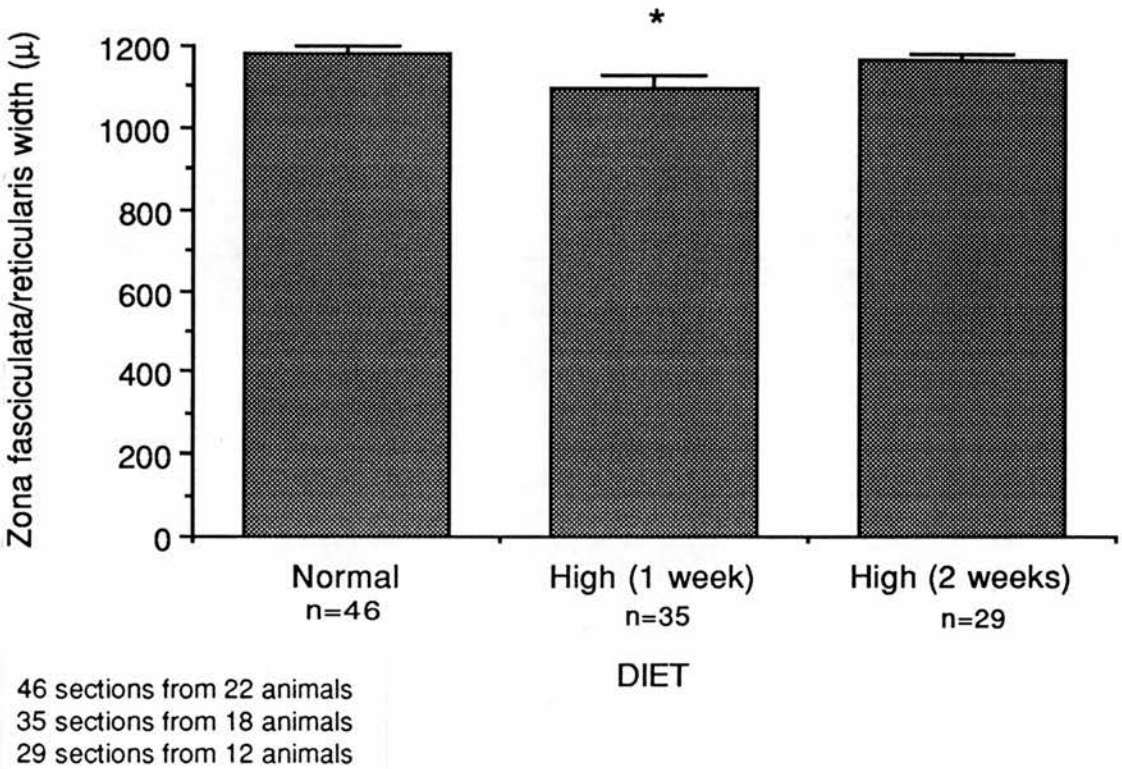


Figure 7.14. **The effect of sodium loading on zona fasciculata / reticularis width.**

Figure 7.15 shows the zona fasciculata / reticularis : zona glomerulosa ratios during the various dietary regimes. The ratios were derived from the means of the width of the zones from each group. The normal ratio ranged from 16.8 to 19.4. Both a low sodium and a 5HTP diet decreased the ratio, whilst a sodium replete diet increased the ratio.

7.5.4. The mechanism of 5HTP stimulated adrenal growth

It is clear that chronic administration of 5HTP, which enhances circulating levels of serotonin, results in an increase in adrenal zona glomerulosa width. However, it is unclear if this is mediated by specific serotonin receptors which increase in number and binding affinity following 5HTP administration in a similar way to the angiotensin II receptor during sodium restriction, or whether it is mediated indirectly by activation of either the renin-angiotensin system or the hypothalamo-pituitary adrenal axis.

In order to investigate this, the zona glomerulosa was measured after administration of 5HTP alone or in the presence of either captopril or dexamethasone. In addition, measurements of PRA, plasma angiotensin II, corticosterone and aldosterone were made, firstly to study any activation of the renin angiotensin system or the hypothalamo-pituitary adrenal axis following 5HTP administration and secondly as a marker to test the efficacy of captopril and dexamethasone in suppressing each of the systems respectively. As controls, plasma angiotensin II and serotonin concentration were measured during altered sodium status, which is already known to cause changes in morphology via the renin-angiotensin system.

Figure 7.16 shows plasma levels of angiotensin II in normal animals and those on sodium deplete and sodium replete diets for 1 week. Angiotensin II concentration increased from 38.6 $\pm$ 5 pM in the normal group to 104 $\pm$ 16.7 pM ($p<0.001$) in the sodium deplete group. Sodium loading caused angiotensin II to decrease to 24.5 $\pm$ 3.5 pM ($p<0.05$).

Figure 7.15.

The effect of the various dietary regimes on the zona fasciculata / reticularis : zona glomerulosa ratio.

DIET/DURATION	ZF/ZR : ZG RATIO
Normal (chow)	16.8
Normal sodium (wholemeal)	19.4
Low sodium (1 week)	11.6
Low sodium (2 weeks)	9.2
High sodium (1week)	19.7
High sodium (2 weeks)	20.3
5HTP (1 week)	12.5
5HTP (2 weeks)	10.0

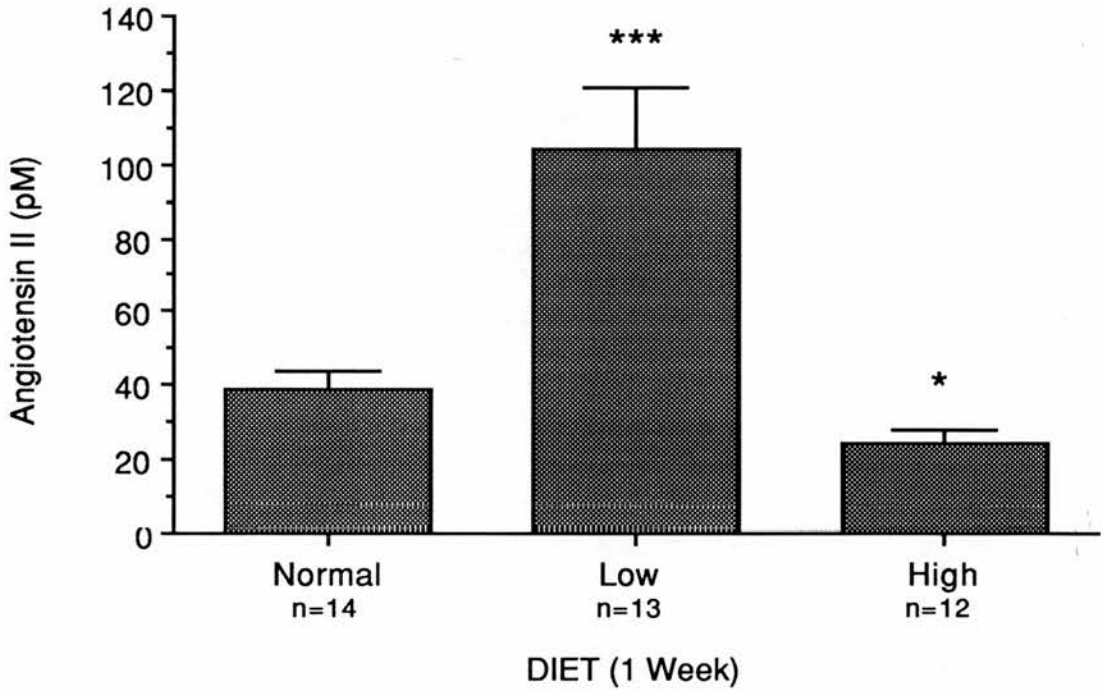


Figure 7.16. **The effect of sodium status on plasma angiotensin II concentration.**

Figure 7.17 shows serum serotonin concentration in animals after 1 week of sodium loading and sodium depletion. Serum serotonin levels showed no significant change with sodium loading. The concentration in the control group was 7.7± 0.6 μM compared with 7.2 ± 0.7 μM in the sodium loaded group. Sodium depletion caused a small but significant decrease in serotonin levels to 6.0 ± 0.6 μM ($p<0.05$).

Figure 7.18 shows PRA following 1 week of captopril or 5HTP plus captopril treatment. PRA increased from 2.6 ± 0.5 ng/ml/hr in the control group to 22.8 ± 3.3 ($p<0.001$) and 20.6 ± 5.1 ng/ml/hr ($p<0.001$) following captopril and captopril plus 5HTP treatment respectively. PRA levels following 5HTP treatment alone are shown in a subsequent illustration.

Figure 7.19 compares the effect of either 5HTP or captopril treatment alone, with 5HTP plus captopril treatment for 1 week on the width of the zona glomerulosa. The width of the zona glomerulosa increased, as before, from 69.6 ± 0.6 μ in the normal (control) group to 86.6 ± 0.9 μ ($p<0.001$) after1 week of 5HTP administration. Captopril treatment alone caused a small but non-significant decrease in width to 66.9 ± 1.9 μ. Captopril plus 5HTP treatment caused a significant increase in width to 75.8 ± 0.8 μ ($p<0.001$), compared to the control group, however the increase was significantly less than that observed with 5HTP alone ($p<0.001$). Representative photographs of adrenal sections (zona glomerulosa) after 1 week of captopril or captopril plus 5HTP treatment are illustrated in figures 7.20 and 7.21.

Figure 7.22 shows plasma corticosterone levels following 1 week of dexamethasone and 5HTP plus dexamethasone treatment. Corticosterone concentration decreased from 483 ± 84 nM in the control group to 4.9 ± 0.8 ($p<0.001$) and 7.9 ± 1.6 nM ($p<0.001$) following dexamethasone and dexamethasone plus 5HTP treatment respectively. Corticosterone levels following 5HTP treatment alone are shown in a subsequent illustration.

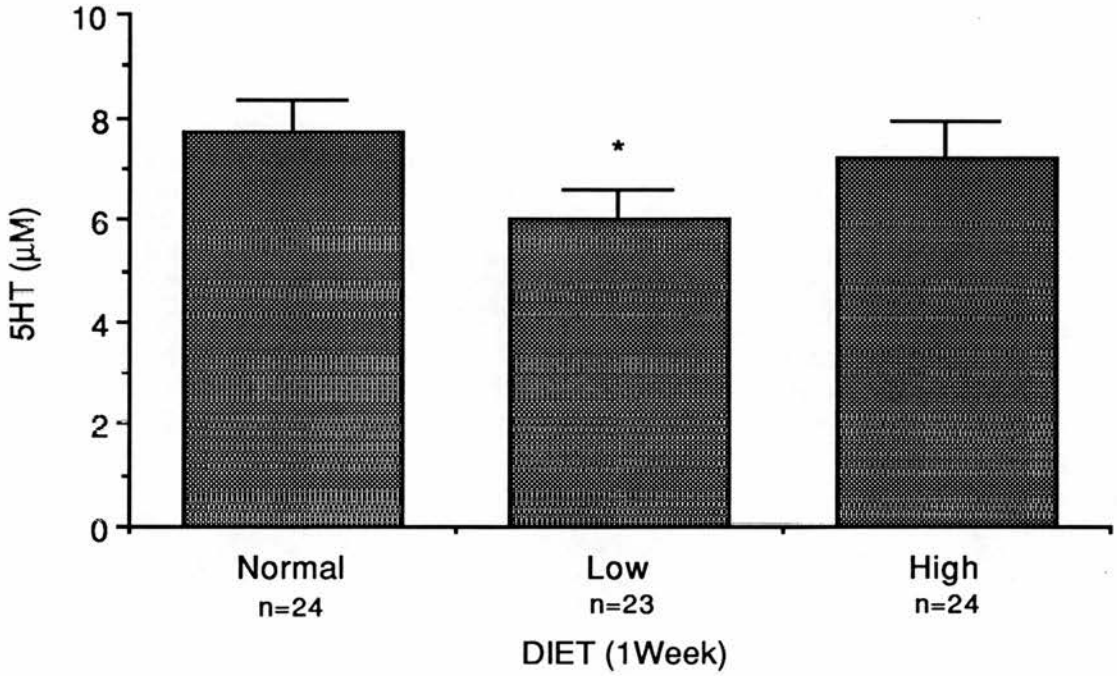


Figure 7.17. **The effect of sodium status on serum serotonin concentration.**

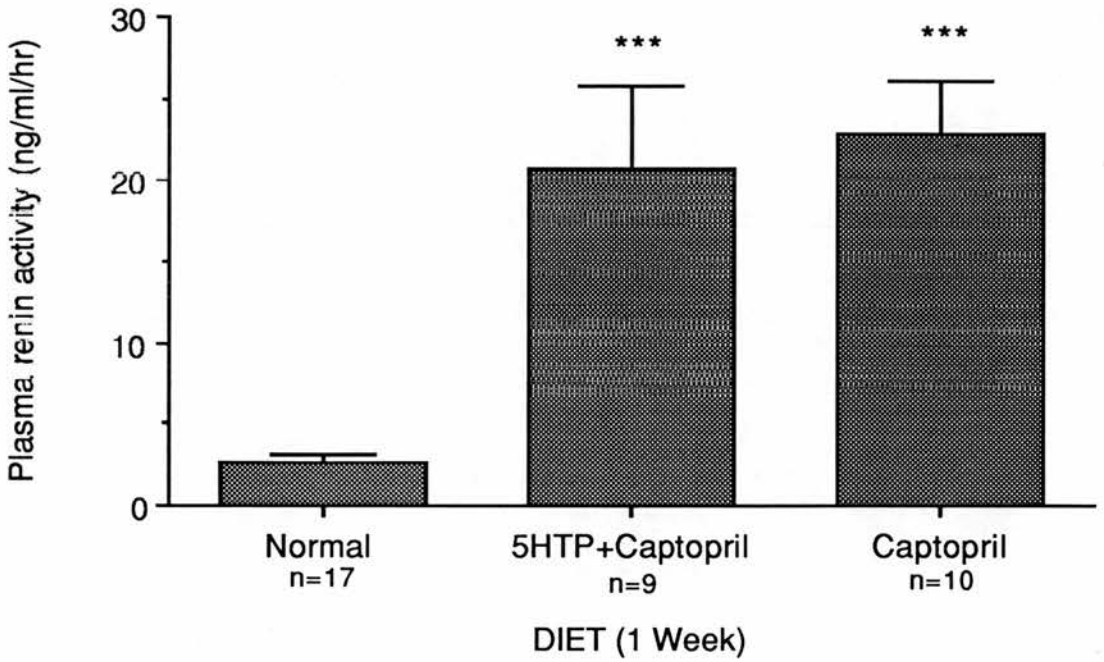


Figure 7.18. **The effect of captopril and 5HTP plus captopril on plasma renin activity.**

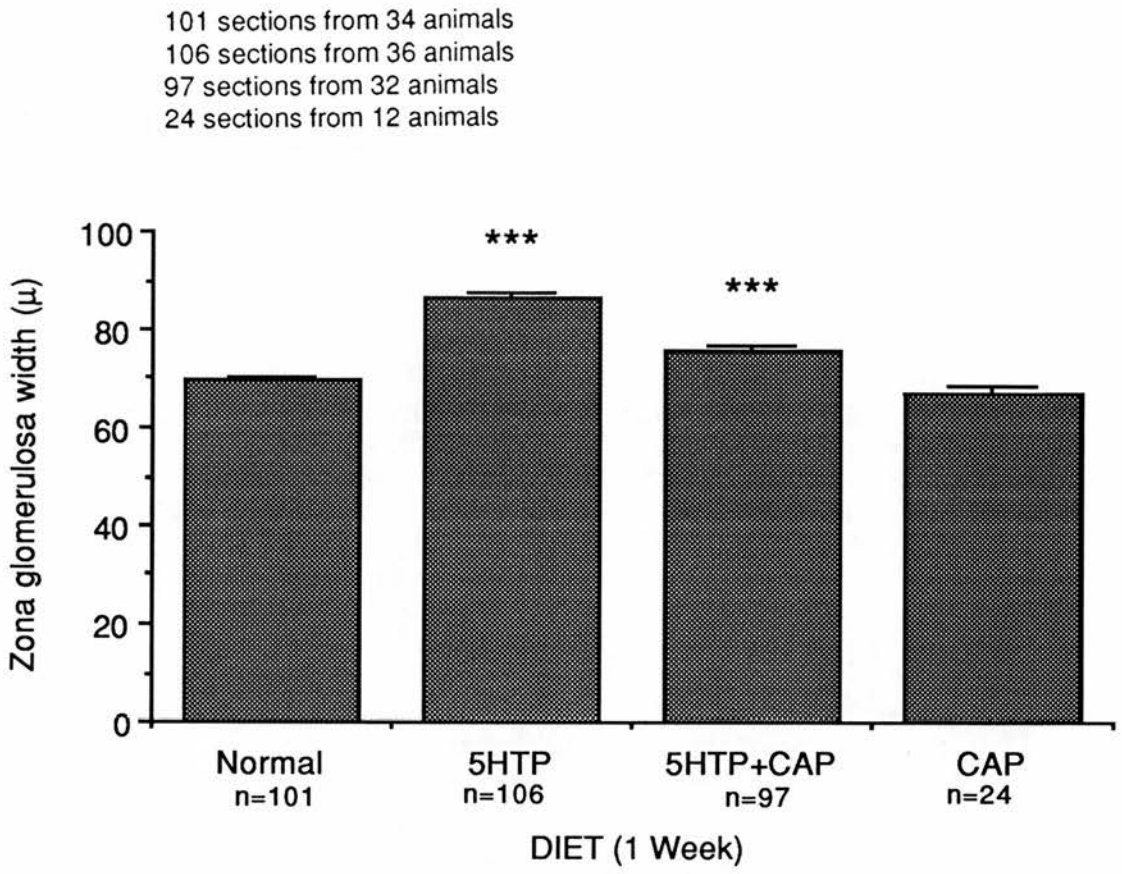
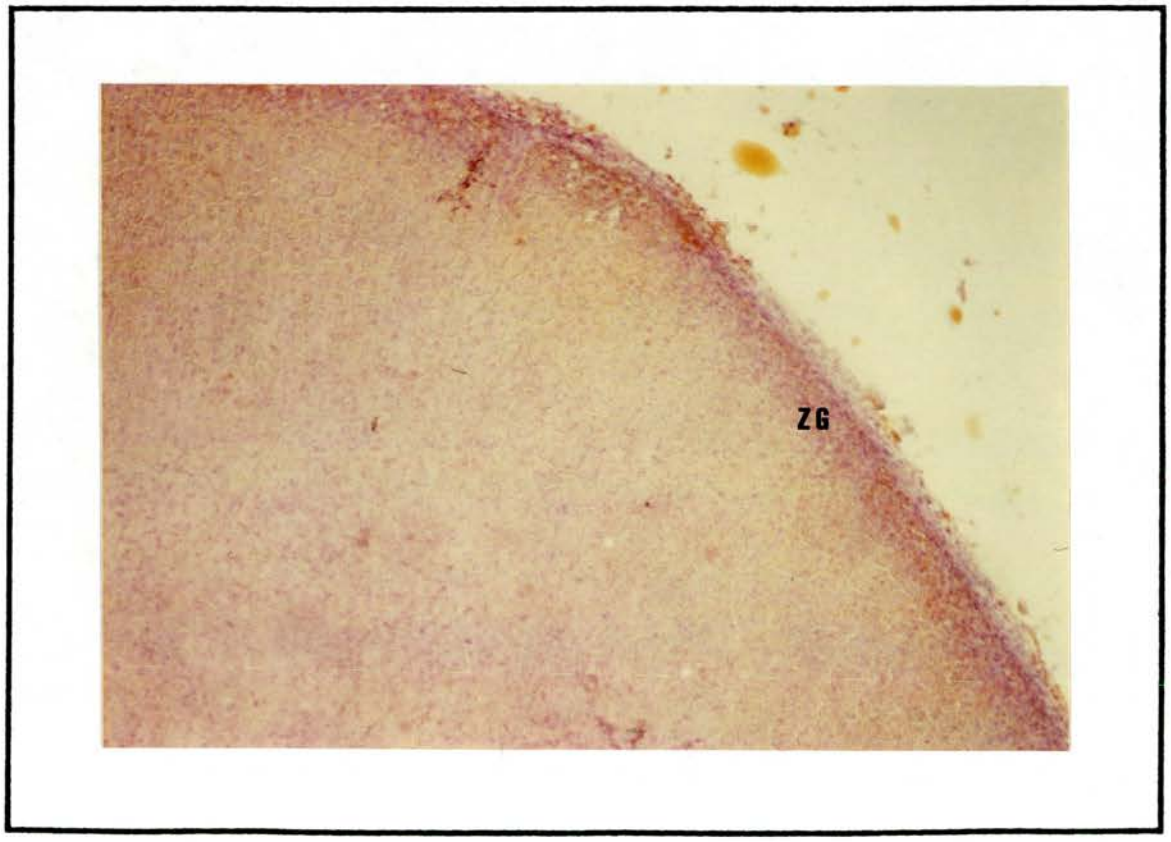


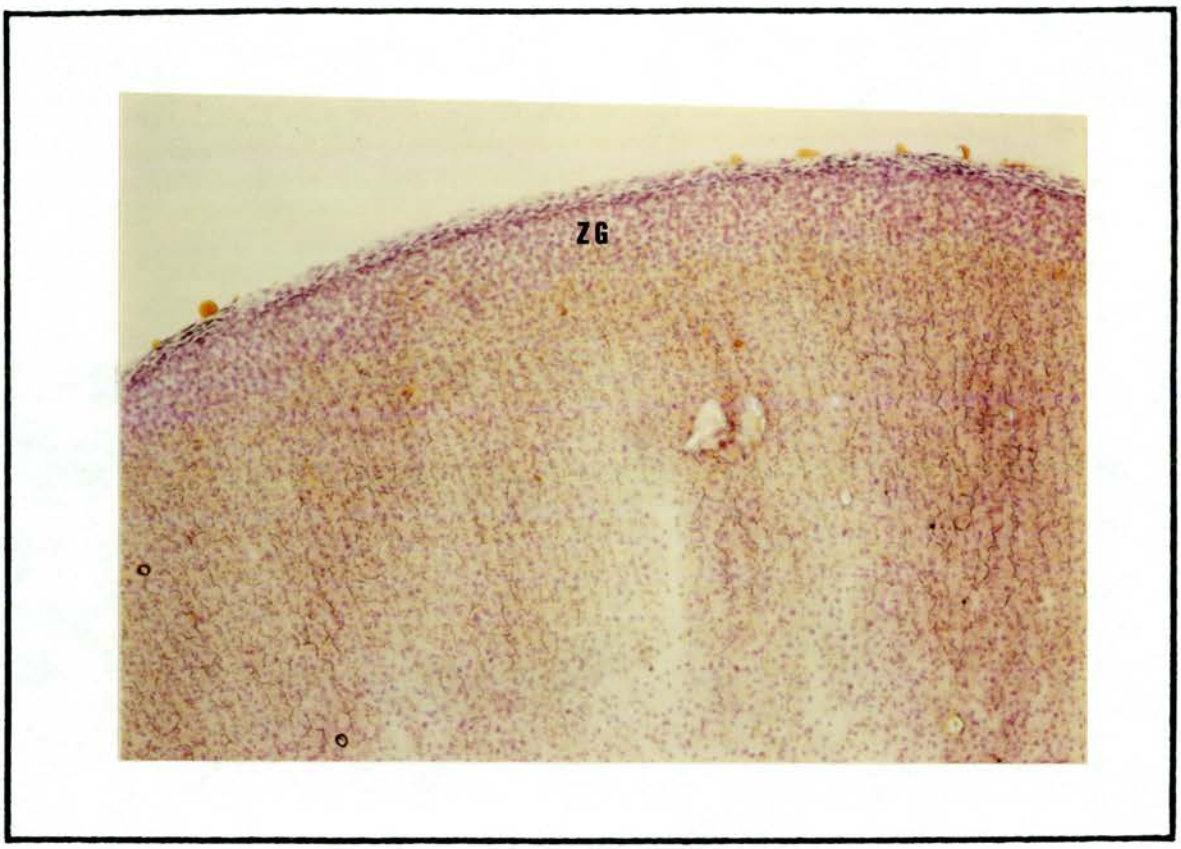
Figure 7.19. **The effect of 5HTP, captopril and 5HTP plus captopril on zona glomerulosa width.**

Figure 7.20.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of captopril treatment.

100 microns





100 microns

Figure 7.21.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of 5HTP plus captopril treatment.

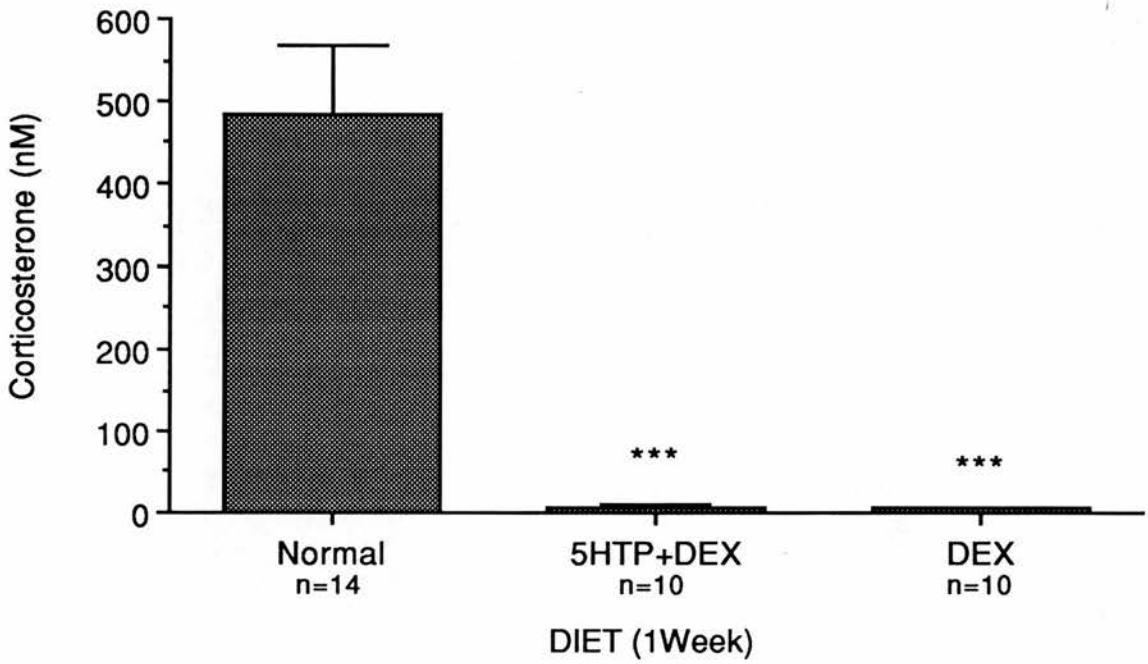


Figure 7.22. **The effect of dexamethasone and 5HTP plus dexamethasone on plasma corticosterone concentration.**

Figure 7.23 compares the effect of either 5HTP or dexamethasone treatment alone, with 5HTP plus dexamethasone treatment for 1 week on the width of the zona glomerulosa. The width of the zona glomerulosa increased, as before, from 69.6 ± 0.6 μ in the normal (control) group to 86.6 ± 0.9 μ ($p<0.001$). Dexamethasone treatment alone caused a significant increase in width to 77.1 ± 1.4 μ ($p<0.001$). Dexamethasone plus 5HTP treatment caused a small but significant increase in width to 93.7 ± 1.4 μ ($p<0.001$) compared to the control group. The increase observed with 5HTP plus dexamethasone was significantly higher than that observed with 5HTP alone ($p<0.001$). Representative photographs of adrenal sections (zona glomerulosa) after 1 week of dexamethasone or dexamethasone plus 5HTP treatment are illustrated in figures 7.24 and 7.25.

Figure 7.26 shows PRA in the control group and after 1 week of 5HTP treatment. 5HTP treatment showed no significant effect on PRA which was 2.6 ±0.6 ng/ml/hr in the control group compared to 2.7 ± 0.8 ng/ml/hr in the 5HTP treated group.

Figure 7.27 shows plasma angiotensin II levels in the control group and those receiving 5HTP for 1 week. 5HTP treatment caused no significant change in plasma angiotensin II concentration, which was 34.5 ± 7.7 pM in the normal group and 40.5 ± 7 pM in the 5HTP treated group.

Figure 7.28 shows plasma corticosterone levels in the control group and after 1 week of 5HTP treatment. 5HTP treatment showed no significant effect on corticosterone concentration which was 484 ± 84 nM in the control group compared with 274 ± 53 nM in the 5HTP treated group.

Figure 7.29 shows plasma aldosterone levels in the control group and after 1 week of 5HTP treatment. 5HTP treatment showed no significant effect on aldosterone concentration

101 sections from 34 animals
106 sections from 36 animals
50 sections from 25 animals
46 sections from 23 animals

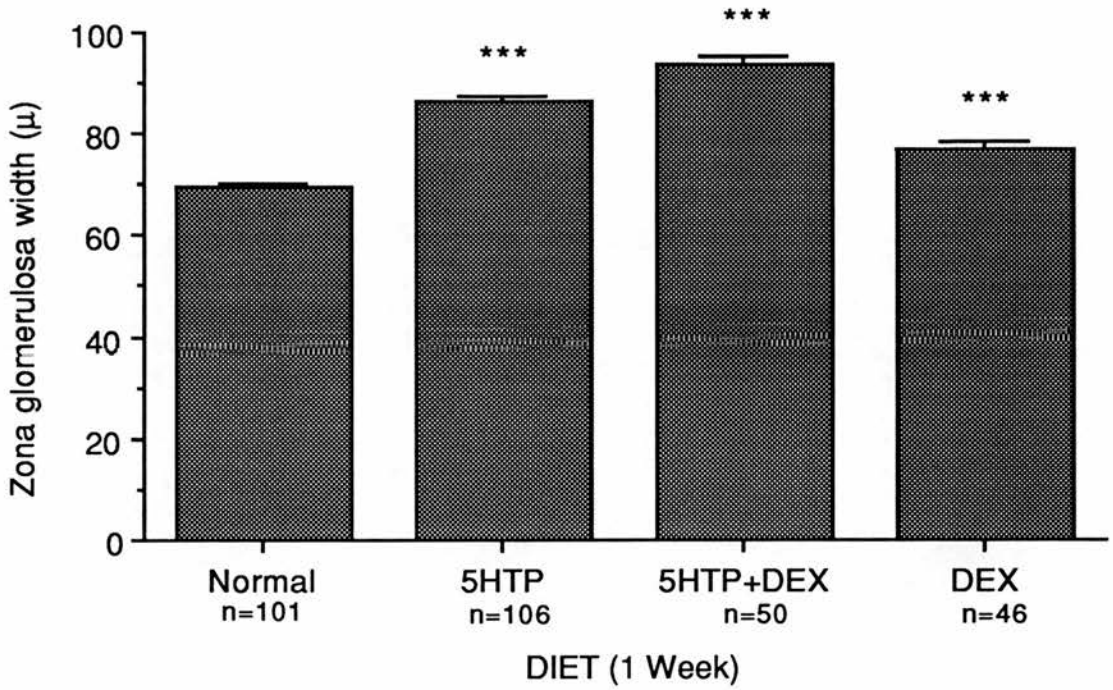
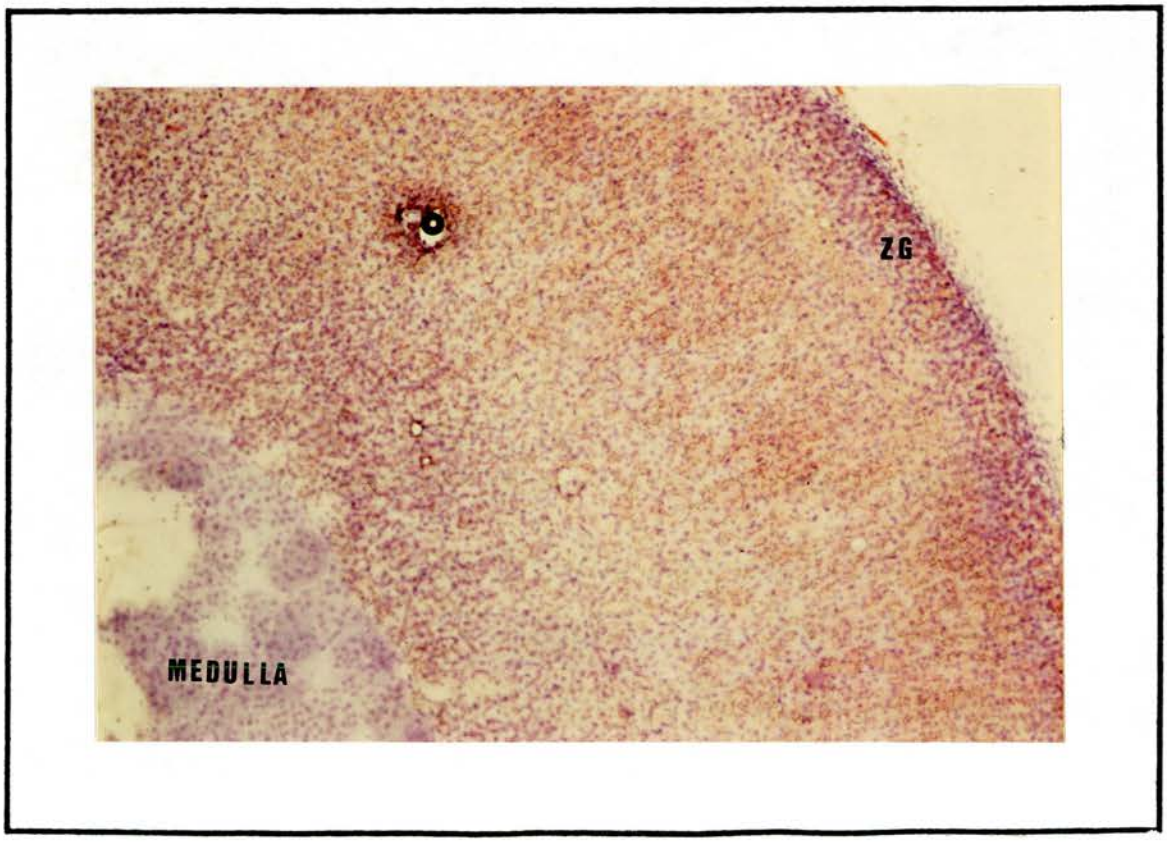


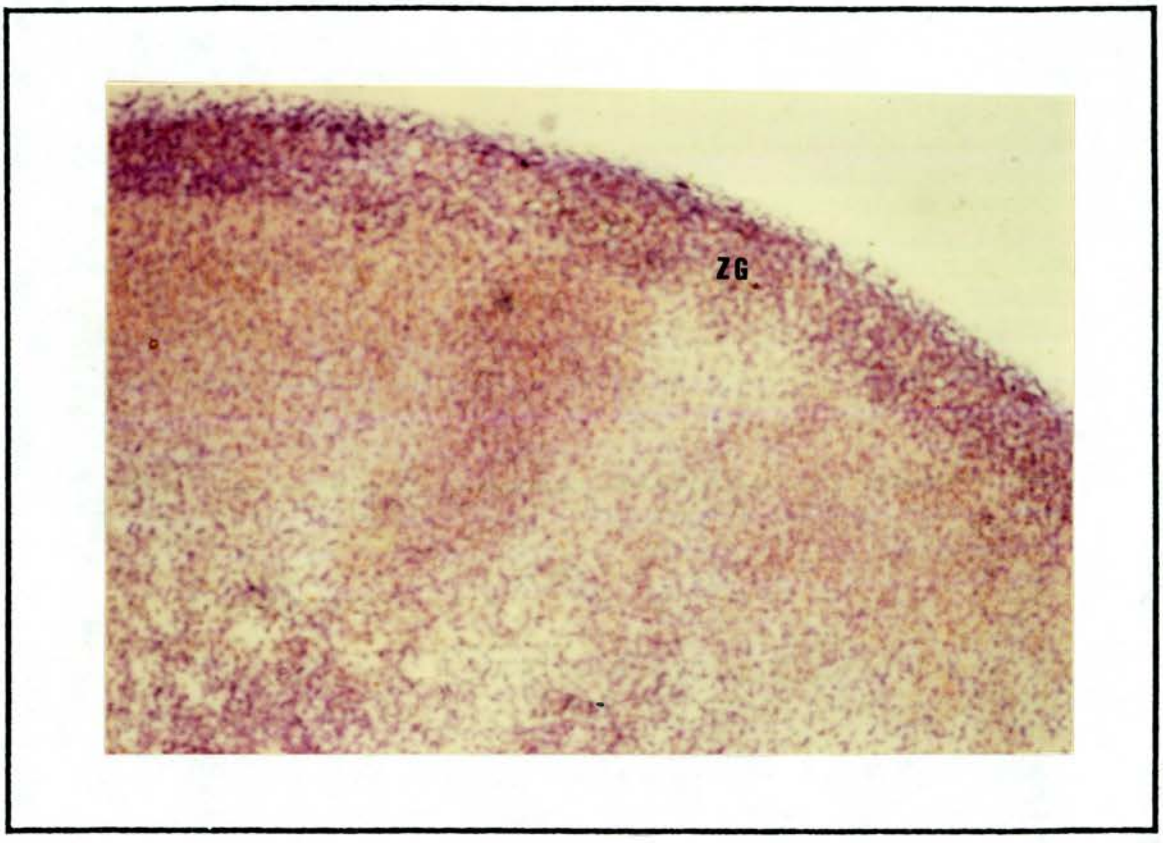
Figure 7.23. **The effect of 5HTP, dexamethasone and 5HTP plus dexamethasone on zona glomerulosa width.**

Figure 7.24.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of dexamethasone treatment..

100 microns





100 microns

Figure 7.25.

A representative photograph of the changes in zona glomerulosa width observed after 1 week of 5HTP plus dexamethasone treatment.

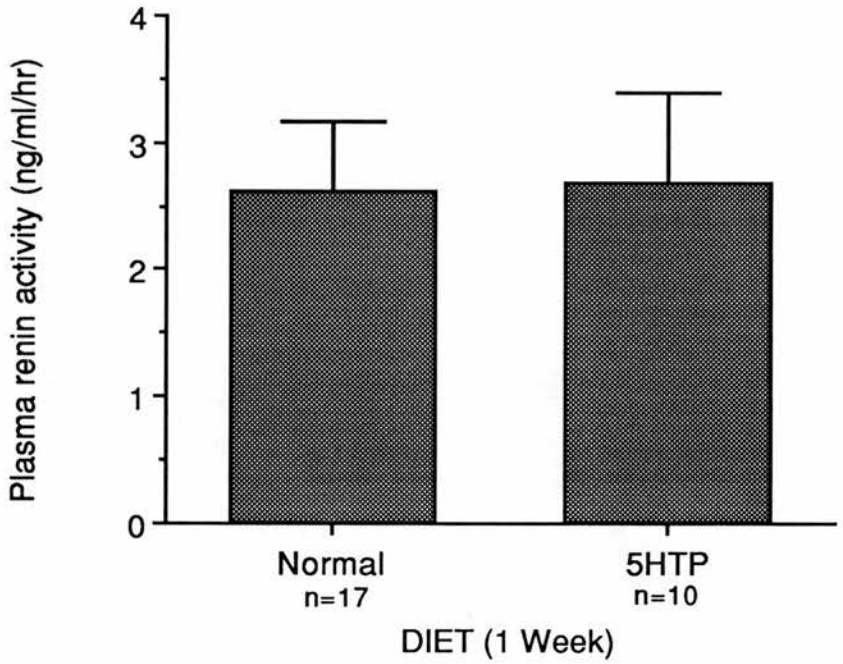


Figure 7.26. **The effect of 5HTP administration on plasma renin activity.**

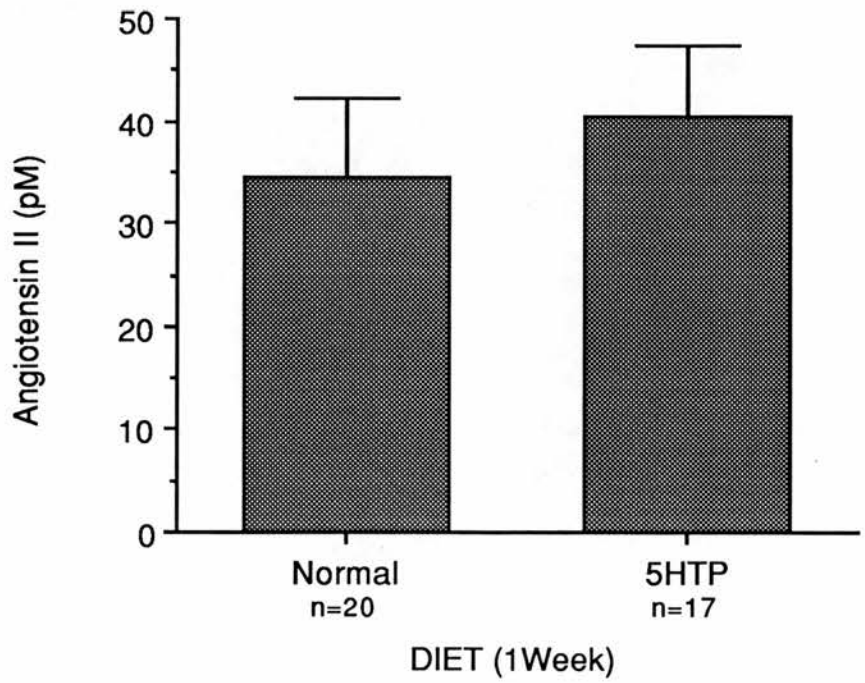


Figure 7.27. **The effect of 5HTP administration on plasma angiotensin II concentration.**

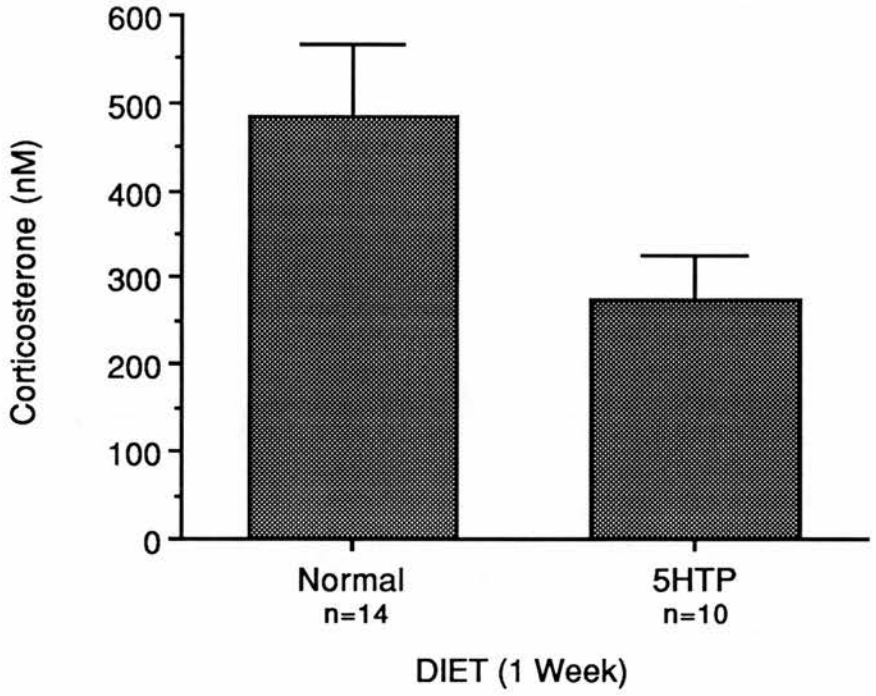


Figure 7.28. **The effect of 5HTP administration on plasma corticosterone concentration.**

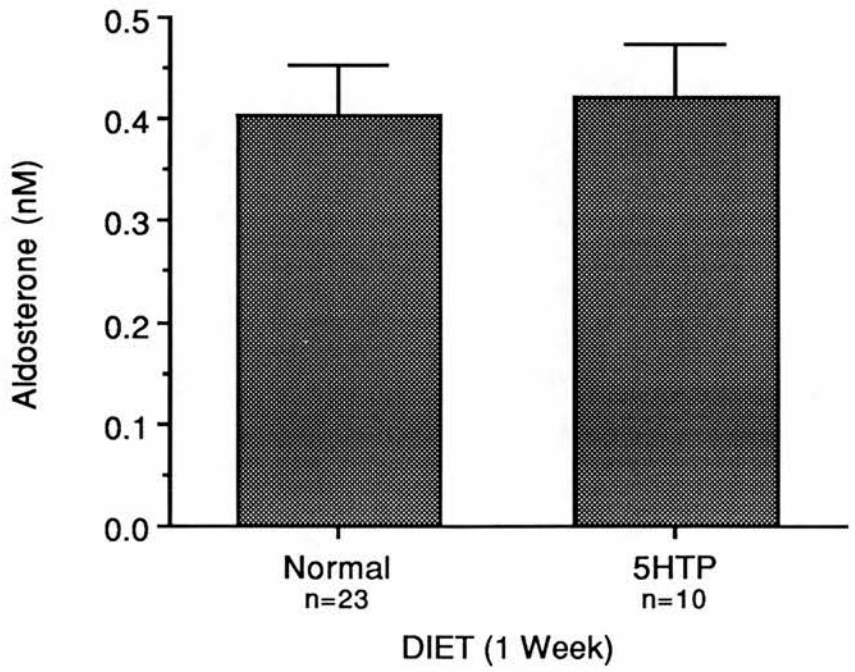


Figure 7.29. **The effect of 5HTP administration on plasma aldosterone concentration.**

which was 0.40 ± 0.05 nM in the control group compared with 0.42 ± 0.05 nM in the 5HTP treated group.

7.6. Discussion

A number of factors which are thought to play either a major or a minor role in the regulation of aldosterone secretion, have been found to have a marked effect on the growth of the adrenal zona glomerulosa. These include sodium depletion, potassium loading, prolactin and α-MSH, which increase the width of the zone, whilst sodium loading, potassium depletion and somatostatin cause a decrease (Deane *et al* 1948, Marx and Deane 1963, Müller and Huber 1969, Rebuffat *et al* 1984, Mazzochi *et al* 1980, 1985, 1986, Robba *et al* 1986). These studies attempted to investigate the effect of chronic elevation of circulating serotonin levels on adrenal growth in animals treated with 5HTP, the precursor to serotonin, for up to 2 weeks. 5HTP was dissolved in the animals drinking water and was made up fresh twice daily to avoid any degradation. The experiments in this chapter were confined to studying the effects of enhanced serotonin status on adrenal growth and hormone secretion. Depletion of circulating levels of serotonin using PCPA, which inhibits central and peripheral formation of serotonin and therefore acts as a serotonin depleting agent, were terminated as the drug caused the animals to become extremely ill after only 2 days of treatment. Depletion of serotonin levels using carbidopa was not considered as it causes a concomitant decrease in the circulating levels of dopamine, which may play a role in the maintenance of adrenal zonal width and make interpretation of the results difficult.

The results show clearly that circulating serotonin levels showed approximately a 3 and 2 fold increase after 1 and 2 weeks of 5HTP treatment respectively. This was accompanied by a marked increase in zona glomerulosa width and a concomitant decrease in zona fasciculata / reticularis width. The zona glomerulosa increased by 20 and 30% and the zona fasciculata / reticularis decreased by 10 and 20% after 1 and 2 weeks of treatment respectively. These changes were compared with those observed during alterations in sodium status. The

sodium diets acted both as a control for the methodology as the results have been documented by a number of groups, and as a marker for the relative potency of each dietary manipulation. Sodium depletion, as expected, caused a 65 and 83% increase in zona glomerulosa width after 1 and 2 weeks respectively. Zona fasciculata / reticularis width showed very little change after 1 week and decreased by 13% after 2 weeks of treatment. Conversely, sodium loading, as expected, caused an 11 and 8% decrease in zona glomerulosa width after 1 and 2 weeks of treatment respectively. The zona fasciculata / reticularis, however, also decreased by 7 and 2% after 1 and 2 weeks of treatment respectively.

The results show clearly that chronic elevation of circulating serotonin results in an increase in zona glomerulosa width and a parallel decrease in zona fasciculata / reticularis width. It cannot be concluded yet whether this is a true hypertrophic effect i.e. an increase in cell division, as observed in the zona glomerulosa following sodium restriction (Race and Green 1955), or indeed a hyperplastic effect leading to an increase in the mass of the glomerulosa cells and changes in their ultra-structure, as observed in zona fasciculata cells following chronic administration of ACTH (Bransome 1968, Nickerson 1975). However, for simplicity the increase in width will be referred to as a "trophic" effect. Comparison of the increase in width with 5HTP and low sodium status indicate that serotonin is not as potent a stimulus for glomerulosa growth as a sodium deplete diet. A number of other studies have demonstrated the mitogenic properties of serotonin, particularly in vascular smooth muscle cells and fibroblasts (Nemecek *et al* 1986, Seuwen *et al* 1988). More recently, melatonin, which is formed from serotonin by two enzymatic reactions, has been shown to stimulate zona glomerulosa hypertrophy and aldosterone secretion, primarily by increasing the volume of mitochondria and smooth ER, which contain the enzymes involved in aldosterone biosynthesis (Tamaoki *et al* 1973, Rebuffat *et al* 1988,)

Although the "trophic" action of serotonin is clear, the mechanism by which it exerts its effects is difficult to establish, and a number of possibilities exist. Firstly, it may act by direct

modulation of specific serotonin receptors located on the zona glomerulosa, which may up-regulate in response to increased serotonin status, therefore increasing in number, affinity and responsiveness to serotonin. This has at least been demonstrated *in vitro*, whereby the responsiveness of aldosterone secretion to serotonin is enhanced in zona glomerulosa cells isolated from animals maintained on a 5HTP diet (Shaikh *et al* 1986), in a similar way to the changes observed in angiotensin II receptors and responsiveness during a sodium deplete diet (Glossman *et al* 1974, Aguilera *et al* 1978, Aguilera *et al* 1980). Alternatively, serotonin may act indirectly to modulate other systems such as the renin-angiotensin system or the hypothalamo-pituitary adrenal axis. In this respect, the "trophic" effect of ACTH on the zona fasciculata is considered to be indirect, and may be mediated by its other actions such as increased blood flow or cyclic AMP secretion (O'Hare and Neville 1973, Marton *et al* 1976, Lewinski and Szkudlinski 1981).

Considering the renin-angiotensin system first, the results of chapter 6 and data from other groups have shown that serotonin can activate the renin-angiotensin system (Zimmerman and Ganong 1980). This would increase circulating levels of angiotensin II, in a similar way as a sodium deplete diet. This chapter and a number of other groups have shown that sodium restriction results in increased angiotensin II levels and zona glomerulosa hypertrophy , resulting directly from an increase in the mitotic index of the glomerulosa cells (Deane *et al* 1948, Peschel and Race 1954). The effects on the inner zones are conflicting, some groups showing no change (Deane *et al* 1948, Müller and Huber 1969), whilst others have shown a parallel decrease (Hartroft and Eisenstein 1957, Blair-West *et al* 1968). The increase in the zona glomerulosa width is associated with an increase in rough and smooth ER, golgi apparatus and mitochondrial surface area, and an increase in the activity of many enzymes (Shelton and Jones 1971, Giacomelli *et al* 1965, Fisher and Horvat 1971, Marx and Deane 1963, Cohen 1965, Kuhn and Kissane 1964, Elema *et al* 1968). In addition to the cellular changes, the aldosterone response to angiotensin II, ACTH, potassium and serotonin is enhanced during sodium restriction, although conflicting reports surround the

enhancement of the response to ACTH (Thorn *et al* 1957, Venning *et al* 1962, Kinson and Singer 1968, Müller 1968, 1970, Müller and Huber 1969, Blair-West *et al* 1970, Vinson *et al* 1983). Therefore, it is possible that serotonin may elicit its effect on the zona glomerulosa by activating the renin-angiotensin system. This hypothesis was tested by comparing the effect of 5HTP treatment alone on zonal width with that of 5HTP plus captopril or captopril alone. In addition, PRA and angiotensin II measurements were made following chronic 5HTP treatment. Successful blockade of the renin-angiotensin system by captopril was monitored by the increase in PRA in the 2 groups receiving the ACE inhibitor. There was no change in PRA in the group receiving 5HTP alone and consequently, there was also no change in angiotensin II levels, in contrast to the results obtained with altered sodium status, which quite clearly causes alterations in the renin-angiotensin system. The zona glomerulosa measurements in this study show that captopril treatment alone caused a small (NS) decrease in width, probably due to the decrease in circulating levels of angiotensin II which maintain the zona glomerulosa width. The "trophic" action of 5HTP was attenuated by the co-administration of captopril with 5HTP. However, the width was not returned to control values, as would be expected if the action of serotonin is mediated solely by activation of the renin-angiotensin system. The attenuating effect of captopril on 5HTP induced growth is most probably attributable to the action of captopril itself on the renin-angiotensin system, rather than the blockade of the stimulatory effect of serotonin on the system, thus causing a similar effect to sodium loading. This has already been documented by Mazzocchi *et al* 1985, who showed that prolonged captopril treatment caused a significant decrease in the volume of zona glomerulosa cells, in addition to the content of nucleii, mitochondria and SER. The effect is almost certainly more pronounced when the zona glomerulosa width is increased beyond the normal range, as with 5HTP treatment. The alternative explanation is similar to that of chapter 6, wherby angiotensin II is required permissively for the trophic action of serotonin on zona glomerulosa growth, probably to maintain both the angiotensin and serotonin receptors. Indeed it has been shown that down-regulation of the angiotensin II

receptor by sodium loading, decreases the adrenal responsiveness to both angiotensin II and serotonin, therefore it is possible that down-regulation of the angiotensin II receptor with captopril may similarly effect the responsiveness of the serotonin receptor *in vivo* and attenuate the trophic effect. Considering the measurements of glomerulosa width following captopril plus 5HTP treatment, together with the PRA and angiotensin II measurements it seems unlikely that activation of the renin-angiotensin system mediates the trophic changes in the zona glomerulosa observed after chronic 5HTP administration.

The activation of the hypothalamo-pituitary adrenal axis by serotonin has been reported by many groups, and the *in vivo* results of chapter 6 also show that stimulation of aldosterone secretion by 5HTP can be blocked with dexamethasone, suggesting involvement of this branch of the CNS. However, these have been primarily acute studies, whereas this study has concentrated on the chronic action of serotonin. It is expected that chronic activation of the hypothalamo-pituitary adrenal axis would result in overproduction of ACTH which is primarily concerned with glucocorticoid secretion and maintainance of zona fasciculata width. Therefore, chronic elevation of ACTH levels by serotonin would presumably increase zona fasciculata width and decrease zona glomerulosa width. The trophic effects of ACTH have been reported by other groups who have shown chronic elevation of ACTH decreased zona glomerulosa width and caused structural disruption leading to the conversion of glomerulosa cells to fasciculata-like cells and the loss of responsiveness of aldosterone secretion to serotonin which is unique to the glomerulosa (Shire and Stewart 1972, McDougall *et al* 1980, Müller 1978). Nevertheless, the role of the hypothalamo-pituitary adrenal axis was studied by comparing the effect of 5HTP administration alone with 5HTP plus dexamethasone or dexamethasone alone. Dexamethasone itself caused a significant increase in zona glomerulosa width. This may be due to removal of ACTH which maintains zona fasciculata / reticularis width, resulting in zona fasciculata / reticularis atrophy and subsequent glomerulosa hypertrophy. A similar effect was shown in pre-pubertal rats, whereby administration of a glucocorticoid resulted in the cessation of adrenocortical growth, and later adrenal atrophy

(Wright *et al* 1974). Similarly the trophic action of 5HTP was enhanced to a small degree in the presence of dexamethasone, again this may be due to zona fasciculata atrophy and concomitant zona glomerulosa hypertrophy caused by the glucocorticoid. These results, together with the corticosterone measurements after 5HTP treatment which show no significant change, suggest that activation of the hypothalamo-pituitary adrenal axis does not mediate the chronic effects of serotonin. It is noteworthy that the corticosterone levels in this particular study were relatively high compared with measurements made in other studies. This is probably indicative of stress levels in the animals preceding cervical dislocation. It is also of interest to note that the corticosterone levels in the 5HTP treated animals are somewhat lower than in the control group, although it is non-significant. This is perhaps due to the decrease in zona fasciculata / reticularis width following 5HTP treatment, as this zone is the major site of corticosterone biosynthesis.

In summary, the results suggest that chronic elevation of plasma serotonin results in zona glomerulosa "hypertrophy", similar to, but not as effective as a sodium deplete diet. The studies carried out were not able to determine if this was a result of increased mitotic activity or an increase in cell volume and ultra-structure, nor did they determine whether serotonin is a direct growth factor like angiotensin II or an indirect factor like ACTH. The effect does not seem to be mediated by activation of either the renin-angiotensin system or the hypothalamo-pituitary adrenal axis. Therefore, it seems possible that the effect may be mediated by discreet alterations in specific receptors for serotonin within the zona glomerulosa, although the possibility that serotonin could stimulate the production of another growth factor (s) cannot be excluded at this stage. It is of particular interest that despite stimulating the growth of the zona glomerulosa, chronic administration of 5HTP, fails to show any significant effect on aldosterone secretion. This is in contrast to other trophic factors such as sodium depletion, suggesting an "escape" mechanism from increased aldosterone secretion. This may be by increasing secretion of another factor which inhibits aldosterone secretion such as ANP, which has been previously reported to increase tritiated thymidine

uptake in cultured zona glomerulosa cells (Horiba *et al* 1985). The stimulatory effect of ANP on mitogenesis indicates that increasing steroid secretion is not a pre-requisite for the trophic action of a hormone. Further studies are required to determine the exact nature of the "trophic" effect of serotonin on the adrenal cortex, and investigate changes in the number and affinity of serotonin receptors within the zona glomerulosa and establish the role of the amine in uncontrolled cell growth as seen in carcinoma of the adrenal gland.

Chapter Eight

General Discussion and Future Studies.

8.1. Discussion

The data obtained from the four chapters suggest, at least in part, that serotonin may play a physiological role in the regulation of mineralocorticoid secretion.

The serotonin antagonist studies provide some, though not conclusive, evidence for the existence of specific serotonin receptors within the zona glomerulosa. These appear to be predominantly $5HT_{1C}$ / $5HT_2$ like receptors. However, the limitations of such studies, measuring end steroid in the presence of drugs and extrapolating these back to the initial receptor event, must be taken into full consideration in the interpretation of the results. Definitive proof of specific serotonin receptors would require a more detailed pharmacological profile, using a more extensive range of antagonists, in addition to molecular studies probing the adrenal cortex for the expresssion of the genes encoding the serotonin receptors, some of which have already been cloned and sequenced in other tissue (Julius *et al* 1988, Fargin *et al* 1988, Pritchett *et al* 1988). Radioligand binding studies in isolated zona glomerulosa cells, using either radiolabelled serotonin or a serotonin antagonist would also be a useful experimental tool for investigating both the type, number and affinity of the serotonin receptors. These studies would also be useful in the investigation of changes in receptor number and affinity during altered serotonin or sodium status or indeed in the hypertensive animal model. Autoradiography with radiolabelled serotonin, using sections of whole adrenal glands could also be used to demonstrate the presence of binding sites and confirm their confinement to the zona glomerulosa. However, attempts at radioligand binding studies in this laboratory, using ^{125}I- serotonin were largely unsuccessful, probably because of the unstable nature of the compound.

The serotonin receptors within the zona glomerulosa, like those of ACTH, appear to be coupled to the adenylate cyclase second messenger system. There is no evidence of PI turnover as observed with angiotensin II. In addition, the presence of extracellular calcium and inward transmembrane flux of calcium seem to be prerequisite for the induction of the cascade mechanism leading to enhanced aldosterone secretion. The role of calcium as a

mediator of the end steroid response in these studies has been monitored by largely indirect methods, using calcium antagonists and chelating agents etc, yielding qualitative results. A more detailed and quantified analysis could have been obtained from fluorescent dye studies, whereby dyes such as Fura-2 permeate the cell membrane and fluoresce intensely on binding of calcium, following stimulation. These can be carried out in both cell suspensions and single cells. In addition, it would be possible to define the source of the calcium, be it extracellular or from intracellular organelles such as the endoplasmic reticulum. Although these type of experiments were not carried out in this laboratory, they have been carried out by a number of other groups who have shown that in vascular tissue there is a large increase in intracellular calcium ion concentration, following serotoninergic stimulation, which is liberated from an intracellular store in response to formation of IP_3. However, the adrenal serotonin receptor does not appear to utilise the PI second messenger system and fluorescent dye studies carried out in bovine cells have shown no change in calcium levels (Capponi *et al* 1987). However, as bovine adrenal cells have no steroidogenic response to serotonin, this is not an unexpected result. Therefore, similar studies using rat zona glomerulosa cells are necessary, and it would also be useful to compare and contrast the adrenal response with that of the vascular system.

Although it seems almost certain that serotonin receptors exist within the zona glomerulosa and these appear to be coupled to a signal transduction mechanism, it is unclear how serotonin within the body acts to stimulate steroidogenesis or play a role in diseases such as hypertension. Unlike ACTH, which acts as a circulating hormone, free serotonin is found in very small, if any, amounts within the circulatory system. This guards against its potentially lethal capacity to mediate vascular permeabilisation and anaphylactic shock. Therefore, it is stored almost exclusively within platelets, bound to serotonectin or in extra-circulatory compartments such as the gut. Within the CNS, serotonin acts as a neurotransmitter and within the vascular system it is released as part of the blood clotting cascade mechanism. As it cannot circulate freely, serotonin cannot act like a classical

endocrine agent, and must therefore act on the adrenal in an alternative way. It is possible that the platelets act as a circulating storage site of serotonin, which can be released locally at the site of the adrenal by a mechanism not involving the blood clotting process. This may be the result of a shape change or momentary adhesion of the platelet to the surface of the zona glomerulosa cells, which are richly supplied with blood vessels (Osim and Wylie 1983). Alternatively, serotonin stored within the chromaffin cells of the adrenal medulla, may exert a paracrinal influence on the zona glomerulosa, although this hypothesis contradicts the preformed idea that adrenal blood flow is unidirectional from the exterior towards the interior of the gland (Vinson *et al* 1985). In this respect it would be interesting to study the effect of total medullectomy on the steroidogenic response to serotonin. A loss of response would indicate that serotonin receptors within the zona glomerulosa are almost exclusively maintained and stimulated by serotonin stored within the medulla. Mast cells are another possible local source of serotonin. These have been localised in the rat adrenal gland in arterioles just as they penetrate the connective tissue of the capsule (Hinson *et al* 1989). Stimulation of serotonin and histamine release by these cells results in increased perfusion and steroid secretion in the isolated perfused rat adrenal gland preparation, suggesting that mast cells may modulate both vascular and secretory responses in the adrenal. As in the CNS, serotonin may also stimulate the adrenal by acting as a neurotransmitter, although it is unclear how innervated the outer zones of adrenal are compared to the highly innervated medulla. Another theory is that the adrenal gland may be capable of synthesising serotonin locally from the circulating precursors 5HTP or tryptophan or indeed by cleaving the amine from its binding protein serotonectin. In addition, a number of other mechanisms may implicate serotonin as a possible causative or exacerbating factor in hypertension. These include decreased uptake capacity and serotonin content of platelets, accelerated platelet turnover and enhanced sensitivity to various agonists, causing release of serotonin.

In addition to the presence of serotonin receptors within the adrenal, the seeming synarchic relationship between the serotonin and angiotensin II receptor is of particular

interest, although the exact nature of the interaction and the receptors involved requires further investigation. A fine equilibrium between free serotonin derived either from platelets or other storage sites and angiotensin II may play a role in the maintenance of normal mineralocorticoid secretion, and any disturbance in this balance may manifest itself in altered adrenal function leading to conditions such as essential hypertension and idiopathic hyperaldosteronism. This inter-relationship may also be important in other diseases such as adrenal carcinoma. This originates from studies which have shown the mas-oncogene contains the genetic sequence for the angiotensin II receptor, whilst the genetic message encoding the $5HT_{1c}$ receptor acts as a proto-oncogene under certain circumstances (Jackson *et al* 1988, Julius *et al* 1989). Carcinoma of the adrenal could therefore be caused or associated with discreet changes in the expression of these two genetic messages, although this theory is purely speculative. Once again, molecular studies investigating the level of expression of the genes encoding the angiotensin II and serotonin receptor in normal and tumourous adrenal tissue would be interesting, as both compounds seem to act as growth factors within the adrenal, as shown by the increase in zona glomerulosa width with sodium depletion and serotonin loading.

The putative interplay between the angiotensin II and serotonin receptor may also be a valuable point of exploitation in the treatment of a number of conditions resulting in mineralocorticoid hypersecretion. Selective serotonin antagonists are relatively new drugs and their therapeutic benefits remain largely unknown. However, the advantages of $5HT_3$ antagonists as anti-emetics in chemotherapy have shown particular promise and the treatment of vascular disorders such as migraine and hypertension with other serotonin receptor based drugs have shown moderate success.

The morphological data clearly show that administration of 5HTP increases zona glomerulosa width. However, it is unclear from the studies if the increase is due to a direct mitogenic action of serotonin or a hyperplastic action, resulting in changes in the cell

ultrastructure and hence cell volume and mass. In addition, the possibility that serotonin could stimulate the secretion of another growth factor(s) cannot be ruled out. Tritiated thymidine uptake studies in cultured glomerulosa cells would confirm a direct mitogenic action of serotonin, which has already been shown in vascular smooth muscle cells and fibroblasts by other groups (Nemecek *et al* 1986, Seuwen *et al* 1988). In addition radioligand binding studies with serotonin or angiotensin II would confirm a direct up-regulatory effect of serotonin on its own receptor, and also if there was a parallel change in the angiotensin II receptor. It is of interest that in contrast to other trophic factors such as sodium depletion or potassium loading, there appears to be an uncoupling of glomerulosa growth and steroidogenic capacity, as shown by the lack of increase in aldosterone secretion with chronic 5HTP treatment. Therefore, if the increase in width is mediated by the direct action of serotonin on the adrenal, it seems there is an "escape" mechanism which prevents a parallel increase in aldosterone secretion. This may be due to a concomitant increase in one of the humoral agents which decrease aldosterone secretion, such as dopamine or ANP. ANP has been shown to enhance tritiated thymidine uptake in cultured glomerulosa cells, indicating that stimulation of steroid output is not always pre-requisite for a hormone to act as a trophic factor (Horiba *et al* 1985). In addition to the thymidine uptake studies, a clearer picture would also emerge by measuring plasma levels of ANP or a number of other possible growth factors such as prolactin or vasopressin, besides PRA and angiotensin II. In addition, the effects of serotonin depletion on adrenal growth would be interesting, although the lack of availibility of a suitable depleting agent which produces no side-effects on the animals or interferes with circulating catecholamine levels make this technically difficult.

The trophic effects of serotonin are also of potential interest in that changes in the concentration of serotonin at the site of the adrenal may result in uncontrolled cell division, as observed in the cancerous state. As already discussed, the genetic material encoding the $5HT_{1c}$ receptor acts as a proto-oncogene, therefore there may be some relationship between uncontrolled adrenal growth as observed in cancer and over-expression of the

genes encoding the serotonin receptor, or an increase in local serotonin concentration at the site of the adrenal. Indeed mast cells have been described in one human adrenocortical tumour (Aiba *et al* 1985).

In addition to the direct actions of serotonin on the adrenal, it seems that serotonin, at least acutely, can activate both the renin-angiotensin system, the hypothalamo-pituitary adrenal axis and steroidogenesis. The central pathway appears to be at least partially responsible for the increase in aldosterone observed following administration of 5HTP in the cannulated rat model and normal circulating levels of angiotensin II are required permissively for the full steroidogenic response. Acutely, it appears that there is a close inter-relationship between the renin-angiotensin system and the hypothalamo-pituitary axis following serotoninergic stimulation and also most probably a direct adrenal effect, due to the failure of captopril or dexamethasone to completely inhibit the aldosterone response. A clearer picture of the relative involvement of each system may have been provided using hypophysectomised and / or nephrectomised animals. Studies giving serotonin, which unlike 5HTP cannot cross the blood brain barrier, would also establish the relative involvement of the centrally mediated effects, assuming that there were no changes in blood pressure or that it would not be immediately metabolised.

In conclusion, serotonin acts in a multi-functional capacity to maintain normal mineralocorticoid secretion, having the ability to act directly at the adrenal cortex or modulate the renin-angiotensin system and the hypothalamo-pituitary adrenal axis. Although further investigations are required to cleary establish the physiological role of the amine, these studies provide a small insight into the potentially large and now unfolding area of research into serotonin and its role in adrenal and cardiovascular homeostasis.

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